PROTEIN VARIATION IN THE MALARIA PARASITE PLASMODIUM FALCIPARUM

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Abstract

Proteins of the asexual, erythrocytic stages of the human malaria parasite Plasmodium falciparum have been analysed by two-dimensional gel electrophoresis (2DGE). Metabolic labelling of in vitro cultures was combined with double label autoradiography in order to compare the proteins of a range of parasite isolates. Nineteen out of one hundred proteins analysed varied electrophoretically in some isolates and thirteen of these proteins were selected as a basis for typing parasite isolates. Sixteen different combinations of variants of these proteins were observed among twenty-one isolates analysed. Fourteen isolates from Thailand and one from the Gambia all had unique protein profiles. Six Papua New Guinea isolates appeared identical to each other but were distinct from the others. The extent and pattern of this intraspecific variation has been considered in relation to the population structure of P. falciparum and to the geographical origin of the isolates. Seven isolates which apparently had more than one electrophoretic variant of certain proteins were provisionally classed as being mixtures of different parasite types. This has been confirmed for one of these isolates (T9). Analysis of several cloned lines derived from T9 identified two types of clone, each possessing only one variant of each protein.

The parasite proteins resolved on 2D gels were also characterised in terms of their molecular weight (MW), isoelectric point,

stage-specificity, glycosylation and antigenicity with the aim of compiling a bank of data about the major parasite proteins. The stage-specific synthesis of proteins during the development of the ring, trophozoite and schizont stages of the erythrocytic cycle was investigated in synchronous cultures. 30 out of 107 proteins analysed exhibited some degree of stage-specificity with the majority of these being synthesised specifically by trophozoites and/or schizonts. Metabolic labelling with ^{3}H-glucosamine identified five intensely labelled glycoproteins, one of which was a high MW basic protein previously shown to vary between isolates. Immune serum was used to identify parasite antigens on 1D and 2D gels. These antigens included a number of strain- and stage-specific proteins. The high MW glycoprotein described above was the major protein recognised by one sample of Thai human serum and was also the target antigen of two monoclonal antibodies analysed.

The results presented in this thesis have been discussed in relation to the relative merits of 2DGE, enzyme electrophoresis and antigen typing for strain differentiation in P. falciparum and in relation to the results of similar 2D gel studies of other organisms. The identity of some of the parasite proteins resolved on 2D gels and the potential value of 2DGE in malaria research have also been considered.

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Declaration

This thesis and the results reported in it are my own work.

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INTRODUCTION

1.1 Malaria and Research

Despite a long history of attempts to eradicate malaria this disease continues to be a major health problem in many parts of the world. In 1982 more than 2000 million people were at risk of infection and there were an estimated 79 million cases of malaria (WHO, 1984a). 90% of these cases occurred in Africa, south of the Sahara where more than one million children may die each year as a result of malaria (WHO, 1979). Tropical regions of India, S. E. Asia (including China) and the Americas also have a high incidence of the disease.

The fight against malaria is based on two main strategies (Pampana, 1969). Foremost are measures directed against the mosquito vectors of the malaria parasite with the aim of reducing the mosquito population, its contact with man and hence the transmission of the disease. Environmental, chemical and biological control methods all play a part but most significant has been the widespread use of residual insecticides such as DDT. The other line of attack is the use of antimalarial drugs to treat or prevent the disease in man and to block its transmission (Bruce-Chwatt et al, 1981). These measures have proved extremely effective in controlling and even eradicating malaria from certain areas of the world, particularly those on the periphery of its range (Bruce-Chwatt, 1980; Wernsdorfer, 1980). However, malaria has maintained its hold over large parts

of the tropics and, despite previously successful control programmes, the last decade has seen a worrying resurgence of the disease in parts of S. E. Asia and S. America (Noguer et al, 1978). This was due partly to political, economic and administrative problems but also to the spread of drug-resistant parasites and insecticide-resistant mosquitos (Bruce-Chwatt, 1979; Wyler, 1983). Thus, it is clear that more effective measures are urgently required in order to curb further resurgences of the disease; to counter the problems of drug- and insecticide- resistance; and to achieve better control of malaria in the highly endemic tropical areas.

Increasing concern about these problems resulted in the expansion of research on malaria. A major stimulus was provided by the World Health Organization in the initiation of their Special Programme for Research and Training in Tropical Diseases (Wernsdorfer, 1976). The immediate, practical aims of this research are to improve existing methods of malaria control and to use them more effectively. However, for the future it will be necessary to develop new ways of controlling this disease and there is much emphasis on the production of vaccines against malaria. In order to achieve both these short and long term objectives it is essential to stimulate research on all aspects of malaria ranging from the ecology of vector mosquitos to the molecular biology of the parasite itself. Only an integrated approach to research and a detailed understanding of the complex interactions between parasite, man and mosquito will ensure the development of new and effective strategies against malaria.

1.2 The Molecular Analysis of the Malaria Parasite

The molecular analysis of the parasite is a key component of current research. Research on the cell biology, biochemistry, antigenic structure and genetics of the parasite is essential for elaborating its basic biology and also has practical implications for the treatment and control of malaria.

Much biochemical research (Sherman, 1979, 1983; Homewood and Neame, 1980) is related to the chemotherapy of malaria. Comparative studies of parasite and host metabolism can identify pathways or enzymes specific to the parasite which could be exploited in the search for new antimalarial drugs (Webster and Whaun, 1982). Elucidating the molecular action of existing drugs and the molecular basis of drug resistance is of value for rationalising the treatment of the disease and for controlling the spread of drug resistance in the field. In addition, the detailed biochemical studies of other aspects of the parasite and its interactions with the host may lead to novel approaches to malaria therapy. For example, analysis of the surface properties of parasites or parasitised cells could allow the development of selective drug targetting techniques.

As mentioned above much hope has been placed in the development of vaccines against malaria (Mitchell, 1984). For this reason the immunology of the disease and the antigenic analysis of the parasite are major areas of current research (Perrin and Dayal, 1982; Deans and Cohen, 1983; Newbold, 1984). It is necessary to

characterise those antigens which can elicit host-protective immune responses during the course of an infection and equally important to identify those which may induce responses which are either ineffective or actually harmful to the host. Such studies will lead to a better understanding of the development of immunity to malaria. The characterisation of parasite antigens may also allow the development of better immunodiagnostic techniques for the disease and this could improve the effectiveness of malaria control programmes.

The genetics of the malaria parasite (Beale, 1980; Walliker, 1983a, 1983b) also has implications for the control of the disease. The reproductive biology of the parasite and its genetic consequences are relevant to the parasite's population structure and to the gene flow within the species. These factors have implications for the occurrence and spread of clinically important characters such as drug resistance or new antigenic types within parasite populations. The organisation of the parasite genome at the molecular level has become the subject of recent research (Hyde et al, 1981; Goman et al, 1982; Coppel et al, 1983, 1984; Kemp et al, 1983; Dame et al, 1984; Hall et al, 1984a; Koenen et al, 1984; Wallach et al, 1984). Study of the structure of genes and the control of their expression is of practical potential for producing parasite proteins, particularly antigens for vaccine trials, by genetic engineering techniques.

The analysis of parasite proteins is an integral part of this molecular research. It is the specific spectrum of structural and

functional proteins present in the parasite which determines its cellular, biochemical and antigenic properties. The interactions between the parasite proteins and host tissue underlie many aspects of the immunology and pathology of the disease. For these reasons much research has focussed on the proteins of the parasite and, as outlined below, this thesis is also concerned with their analysis.

The main objectives of the work presented in the following chapters are to characterise the major protein products of the human malaria parasite Plasmodium falciparum and to investigate the extent and significance of protein variation within this species. The analysis of intraspecific variation is of value in two ways. Firstly, it can provide information about the genetics and population structure of the species and, secondly, it can be used to identify and characterise particular strains of parasite. In order to put this work into perspective, the biology of the malaria parasite is outlined in the following section and previous studies on protein analysis and strain variation in P. falciparum are reviewed in Sections 1.4 and 1.5 respectively.

1.3 The Cell Biology of Plasmodium falciparum

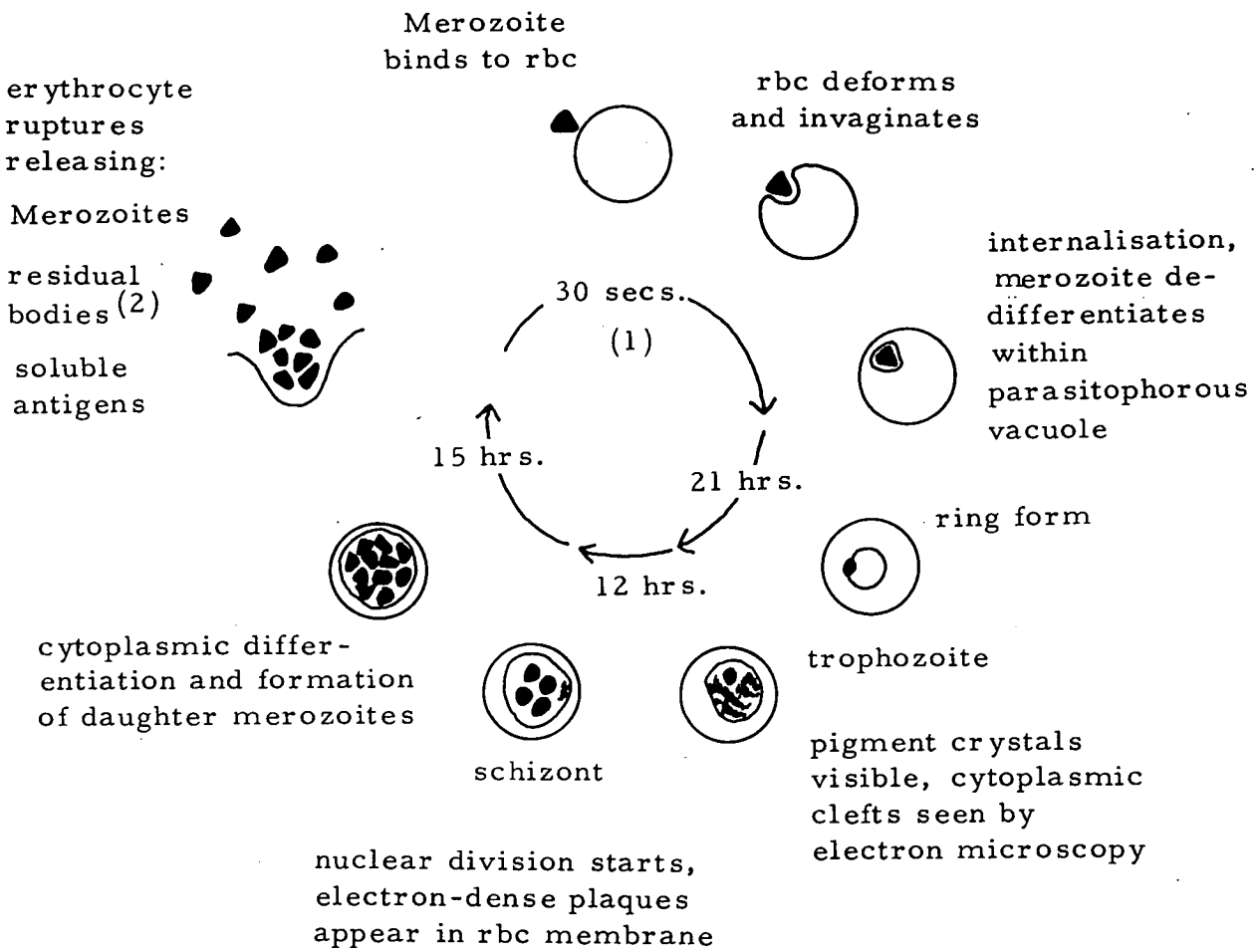
P. falciparum is one of four species of malaria parasites which infect man. However, it causes the most severe form of the disease and is responsible for much of the mortality due to malaria. The life cycle, biology and morphology of this parasite have been described in detail by Garnham (1966), Coatney et al (1977) and

Aikawa (1977). In addition to its growth and differentiation within the vector mosquito, the parasite develops through three morphologically and functionally distinct phases in the human host. The first of these is the infection and replication within cells of the liver (exo-erythrocytic development); the second is a cycle of multiplication within erythrocytes in the blood (the erythrocytic cycle); and the third is the intra-erythrocytic development of the sexual, transmissible stages of the life cycle which are infective to feeding Anopheline mosquitos (gametocytogenesis).

It is the proliferation of parasites in the blood which causes the symptoms and pathological consequences of the disease in man. These parasites are also more accessible to analysis than are the liver or insect stages of development and for these reasons the erythrocytic parasites have been the subject of much malaria research. Detailed biochemical and molecular analysis of these stages has been made possible by the successful development of an *in vitro* culture system (Trager and Jensen, 1976; Haynes *et al*, 1976) and of methods for growing parasites en masse (Jensen *et al*, 1979; Trager, 1979), producing cloned lines (Rosario, 1981; Trager *et al*, 1981) and synchronising parasite development (Lambros and Vanderberg, 1979; Kilejian, 1980a).

The erythrocytic cycle of development is shown in fig. 1.1. The only extracellular stage of the cycle is the merozoite. This is short-lived and rapidly invades the host erythrocyte by a complex series of interactions (Aikawa *et al*, 1978; Aikawa and Miller, 1983).

Fig. 1.1 Erythrocytic schizogony in P. falciparum



(1) Developmental times based on Dvorak et al (1975) and Freeman and Holder (1983 c).

(2) Residual bodies = pigment-containing vacuoles, membrane vesicles, endoplasmic reticulum.

Invasion leaves the merozoite within a vacuole bounded by a membrane derived from that of the erythrocyte (the parasitophorous vacuole membrane). Here the merozoite dedifferentiates losing some of its organelles (e.g. the rhoptries, micronemes and pellicular membranes). The young intracellular parasite is then seen as the typical ring form (fig. 6.1) with its distinct dot of chromatin and narrow ring of cytoplasm.

The full cycle of intra-erythrocytic development takes approximately 48 hours. During this time the parasite increases in size and complexity. The central 'vacuole' of the ring form is replaced by cytoplasm and granules of malaria pigment, derived from the breakdown of haemoglobin, become visible as the parasite develops into a trophozoite. 35-40 hours into the cycle, nuclear division is initiated and during subsequent development of the schizont (fig. 6.1) the characteristic merozoite organelles appear and cytoplasmic partitioning occurs. The mature schizont contains 10-25 uninucleate, daughter merozoites. Rupture of the infected erythrocyte releases these merozoites to initiate a new round of invasion and multiplication. Rupture also releases residual parasite material and soluble antigens into the extracellular medium.

The morphological and ultrastructural changes which characterise the intracellular development of the parasite are paralleled by events at the molecular level. The sequential pattern of gene expression controlling growth and differentiation is reflected in the stage-specificity of mRNA species (Hyde et al, 1984) and in the changing

spectrum of proteins synthesised during the erythrocytic cycle (Kilejian, 1980a; Perrin and Dayal, 1982; Deans et al, 1983b). The onset of schizogony in particular is characterised by marked changes at the molecular level: many new proteins are synthesised, schizont-specific antigens appear (Kilejian, 1980a; Brown et al, 1982) and new metabolic activities, including the synthesis of DNA, become evident (Vial et al, 1982; Gritzmacher and Reese, 1984). Furthermore, the developing schizont induces striking changes in the structure and properties of the host erythrocyte (Howard, 1979). Changes in ultrastructure include the appearance of membrane-bound clefts within the cytoplasm and electron-dense plaques (or knobs) on the surface of the infected erythrocyte (Aikawa, 1977). Other modifications include the presence of new surface antigens (Leech et al, 1984a), altered permeability and transport properties (Kutner et al, 1982; Ginsburg et al, 1983) and decreased deformability (Cranston et al, 1984).

The parasite has evolved several strategies for surviving within its human host. The predominantly intracellular existence of the parasite is one obvious way of avoiding host defences. In addition, trophozoite- and schizont-infected erythrocytes disappear from the peripheral circulation and are sequestered in the capillaries of such organs as the heart, brain and bone marrow (Miller, 1969; Luse and Miller, 1971) due to binding between the knobs on the surface of the erythrocytes and cells of the vascular endothelium. This is thought to benefit the parasite by minimising its contact with

sites of anti-parasitic activity, notably the spleen, at the stage in the cycle when many parasite-induced modifications become detectable. As well as physically evading the host defence system the parasite may contend with it biochemically. The release of soluble parasite antigens into the serum may have a role to play in blocking anti-parasite immune responses (Cohen, 1976) and there is evidence soluble parasite factors may be involved in stimulating the over-production of non-specific immunoglobulin which commonly accompanies malaria infection (Brown, 1976; Greenwood et al, 1979; Gabrielsen and Jensen, 1982). The humoral immune response of the host is directed against two main targets. The first of these is the merozoite which is the only extracellular stage of the erythrocytic cycle and is thus accessible to attack. Anti-merozoite antibodies can block invasion by agglutinating merozoites (Miller et al, 1975) or by inhibiting binding to red blood cells (Epstein et al, 1981) and have been implicated in protective immunity (Jensen et al, 1984). The second target is the surface of the infected erythrocyte itself (Langreth and Reese, 1979; Mendis et al, 1983) and protection could be mediated via antibody-dependent cellular cytotoxicity reactions (Brown and Smalley, 1980) or by blocking the binding of infected erythrocytes to vascular endothelium (Udeinya et al, 1983). There is also evidence that intracellular parasites are a target of protective immune responses (Jensen et al, 1982, 1983). Even when effective responses are mounted by the host, the survival of parasites is thought to be promoted by two factors. One is the

phenomenon of antigenic variation (Hommel et al, 1982, 1983) whereby the antigenic type of the parasite can change over the course of an infection. The other, which is important for the survival of the parasite as a species, is the existence of a diversity of antigenic types within the parasite population. Thus, immunity to one parasite type may not confer protection against subsequent infections by other strains.

1.4 The Analysis of the Proteins of P. falciparum

The analysis of parasite proteins has largely been directed towards the characterisation of functionally important proteins. These can be classed in three main groups: enzymes, antigens, and other proteins interacting directly with host cells. The relevance of enzyme analysis for the chemotherapy and control of malaria has already been mentioned (Section 1.1) and will not be considered further.

The antigenic analysis of the parasite has been the subject of a large volume of research (reviewed by Perrin and Dayal, 1982; Deans and Cohen, 1983; Newbold, 1984; WHO, 1984b). The main aim of this work is to identify antigens which can induce host-protective immune responses in vivo. Two approaches have been used to pursue this objective. One is to determine which parasite products are recognised by antibodies in serum samples collected from endemic areas, infected individuals, experimental animals etc. and then to correlate the immune responses to specific antigens with the immune status of the donor. In this way a number of antigens

have been identified which may be involved in the development of immunity (Reese, Motyl and Hofer-Warbinek, 1981; Perrin et al, 1981a; Brown et al, 1982b, 1983a). The second approach is an empirical one: antisera or monoclonal antibodies against defined antigens can be used to test for anti-parasite activity in vitro (Perrin et al, 1981b; Schofield et al, 1982; Saul et al, 1984). In addition, purified or semi-purified preparations of parasite antigens can be used in trial vaccination procedures in experimental animals or volunteers. Some progress has recently been made in identifying possible protective antigens in this way (Hall et al, 1984a; Dubois et al, 1984; Perrin et al, 1984a, 1984b).

Proteins which interact directly with host cells are of vital importance to the parasite's development and survival within the host and may themselves be the target of protective immune responses. Examples of such proteins are those involved in the invasion of erythrocytes by merozoites. There is much interest in identifying the merozoite components which are involved in the recognition, attachment and invasion of the host erythrocyte (Jungery et al, 1983; Perkins, 1984). Similarly the parasite components present in the knobs of infected erythrocytes which lead to specific binding to vascular endothelium in vivo have also been investigated (Kilejian, 1979; Leech et al, 1984b; Aley, Sherwood and Howard, 1984). Finally, parasite products which have a direct effect on cells of the host immune system must be identified. Components released into the medium of in vitro cultures have been shown to be mitogenic,

causing polyclonal activation of lymphocytes *in vitro*. One parasite product which causes this effect has been partially characterised (Greenwood *et al*, 1979).

Although all these classes of parasite protein can be identified on a functional basis - whether by their enzyme activity, reaction with antibodies or specific binding assays - it is useful to define them more fully in terms of their other biochemical and biological properties. Traditionally, gel electrophoresis techniques have been employed to determine the molecular weight or isoelectric point of such proteins. One-dimensional polyacrylamide gel electrophoresis (PAGE) combined with metabolic or surface-labelling of parasite proteins has been widely used as a means of characterisation. For example, this technique has been used to identify merozoite components (Heidrich *et al*, 1983), glycoproteins (Kilejian, 1980a; Perkins, 1982) and to analyse the stage-specific synthesis and processing of parasite proteins (Deans *et al*, 1983b; Myler, Saul and Kidson, 1983). However, one-dimensional techniques are not totally satisfactory. Only 30-40 polypeptide bands can be easily resolved which is only a small proportion of the expected number of proteins present in a single living cell. Furthermore, estimates of the molecular weight (or isoelectric point) of specific polypeptides may vary depending on the electrophoretic conditions used. Thus, it may be difficult to identify proteins unambiguously by such techniques and also to make accurate comparisons of results from different laboratories. Valuable

information about particular parasite proteins may therefore be lost or not interpreted correctly and one solution to this problem is to employ a two-dimensional gel electrophoresis (2DGE) technique to analyse parasite proteins. The system developed by O'Farrell (1975) uses isolectric focusing in the first dimension to separate polypeptides on the basis of their charge and then SDS-PAGE in the second dimension to separate on the basis of molecular weight. Since polypeptides are separated on the basis of two independent parameters, the resolution obtained is very high and several hundred polypeptide spots may be identified. By using such a technique it is possible to characterise minor parasite components and to identify many protein products with certainty. 2DGE has been used by Tait (1981) to compare the proteins synthesised by different P. falciparum isolates; by Brown et al (1982b, 1983a, 1983b) and Anders et al, (1983) to characterise the antigens recognised by immune sera and by Howard and Reese (1984) to identify merozoite components.

1.5 Strain Variation in P. falciparum

There is considerable intraspecific diversity within P. falciparum. Early reports of strain-specific differences were based on studies of patients infected therapeutically with malaria and of primates infected experimentally. Variation was observed in the duration of exo-erythrocytic schizogony (Garnham, 1966); the severity of infection (James et al, 1932; Jeffery and Eyles, 1954); sensitivity to antimalarial drugs (James et al, 1932; Shute and

Maryon, 1954); and infectivity to mosquitos (Jeffery et al, 1950; Shute and Maryon, 1954; Collins et al, 1963 and 1964). The existence of antigenically distinct types of P. falciparum was also first suggested by in vivo observations. Patients reinfected with the same strain of parasite developed less severe clinical symptoms than those infected with heterologous strains (Jeffery, 1966) and experimentally induced immunity in monkeys showed some degree of strain specificity (Sadun et al, 1966; Voller and Richards, 1970).

More detailed studies have been carried out on the erythrocytic parasites directly by using samples of infected blood or parasites from in vitro cultures. At the cellular level, differences have been described in their ultrastructure (Aikawa and Ward, 1974; Trager et al, 1981) drug sensitivity (Richards and Maples, 1979; Thaithong and Beale, 1981; Thaithong et al, 1983); growth in "immune" serum (Wilson and Phillips, 1976; Reese et al, 1981; Brown et al, 1983b; Cowen et al, 1984) and ability to produce infective gametocytes in vitro (Ponnudurai et al, 1982; Bhasin and Trager, 1984). At the molecular level, electrophoretic analysis of enzymes (Carter and McGregor, 1973; Carter and Voller, 1975; Sanderson et al, 1981; Thaithong et al, 1981), and the characterisation of other proteins (Tait, 1981) and antigens (McBride et al, 1982; Leech et al, 1984a; Wilson and Ling, 1979) has revealed further heterogeneity. Variation has also been detected in the structure of ribosomal RNA (Vezza and Trager, 1982) and in the organisation of repetitive DNA sequences within the genome (Goman et al, 1982).

Variation in such features of the malaria parasite as its virulence, drug sensitivity and infectivity to mosquitos is clearly of great relevance to the therapy, epidemiology and basic biology of the disease. However, it is often difficult to measure these characters reproducibly and to compare different strains accurately. Furthermore, the genetic basis of such phenotypic variation is often unknown. Thus, this type of variation is only of limited value for purposes of characterising strains and investigating the genetics and population structure of P. falciparum.

In contrast, variation in molecular characters is relatively simple to analyse and to interpret. Molecular markers are generally stable, reproducible characters which directly reflect differences in parasite genotypes. For these reasons much research has been directed towards the molecular characterisation of different strains of parasite particularly with respect to their enzymes, antigens and proteins (Walliker, 1983b). The main results of this work are outlined below in relation to the extent of the intraspecific variation detected, its value for strain typing and its relevance to geographical variation within P. falciparum.

Enzyme Variation. The electrophoretic analysis of parasite enzymes has identified two or more variant forms of six different enzymes. For four of these enzymes one variant form is commonly found and other variants are detected only rarely. The other two enzymes - glucose phosphate isomerase (GPI) and lactate

dehydrogenase (LDH) - have two variants which occur frequently (Walliker, 1983a). The small range of enzymes available and the predominance of particular enzyme variants means that enzyme typing is of limited value for differentiating between individual strains of P. falciparum. However, as enzyme variants are good genetic markers, differences in their frequency of occurrence in different regions could provide evidence for geographical variation or sub-speciation within P. falciparum. Thus enzyme typing has been used more extensively for screening infected blood samples, or cultures derived from them, from populations in endemic areas - notably The Gambia, W. Africa (Carter and McGregor, 1973; Carter and Voller, 1975; Sanderson et al, 1981) and S.E. Asia (Thaithong et al, 1981; Myint-Oo et al, 1984). In general, the same enzyme forms have been found in parasites from widely separated geographical areas. However, differences in the relative frequencies of some enzyme variants have been noted. For example, only one form of LDH (LDH-1) has been identified in isolates from Thailand, whereas a second variant (LDH-2) has been found in some isolates from Burma and, at a higher frequency, in African isolates. Although not definitive, these results suggest that some regional variation may exist both between and within different continents.

Antigenic Diversity. Considerable antigenic diversity has been shown to exist in P. falciparum. The most diverse class of parasite antigen which has been analysed is that of the soluble, heat-stable

S-antigens found in the serum of some acutely infected individuals and also synthesised by in vitro cultures (Wilson et al, 1969; Winchell et al, 1984). More than eighteen different antigenic specificities have been detected and since these antigens are stable, reproducible markers of parasite strains this diversity makes them of great potential value for strain characterisation (Wilson, 1980). At present, however, such serotyping is dependent upon the availability of suitable polyspecific antisera from infected individuals or experimental animals.

Other parasite components have been shown to differ antigenically. A number of monoclonal antibodies have been produced which react with only a limited set of P. falciparum isolates (McBride et al, 1982; Schofield et al, 1982). Characterising strains by their reactions with a battery of different monoclonal antibodies forms the basis of an excellent strain typing system. McBride et al (1982) showed that many cultured isolates could be distinguished from each other by the particular combination of monoclonal antibodies with which they reacted.

The serotyping of P. falciparum for S-antigens (Wilson et al, 1969; Wilson, 1980) and the screening with strain-specific monoclonal antibodies (McBride et al, 1982; Schofield et al, 1982; McBride et al, 1984; Knowles et al, 1984) has demonstrated more intraspecific heterogeneity than is apparent from enzyme typing data. Even isolates from the same location can exhibit a wide range of antigenic types. However, as with enzyme variants, the same

antigen types have been found in isolates from widely separated countries and as yet there is no good evidence for distinct, geographically separated parasite populations.

Protein Variation. The majority of reports of protein variation among parasite isolates have related to proteins of known function or identity such as enzymes (see above) or antigens. The S-antigens, for example, have been shown to be biochemically, as well as antigenically, heterogeneous varying in both isolectric point and molecular weight (Wilson and Ling, 1979; Winchell et al, 1984; Anders et al, 1983). Electrophoretic variation has also been detected in a parasite protein recognised by a strain-specific monoclonal antibody (Hall et al, 1984b; Newbold et al, 1984) and in the antigens associated with strain-specific binding to endothelial cells (Leech et al, 1984a). In addition, strains which lack knobs on the surface of parasitised erythrocytes, lack a specific histidine-rich protein synthesised by "knobby" strains (Kilejian, 1979). Until recently, however, there had been no attempt to systematically search for variation among the whole array of parasite proteins regardless of function. Tait (1981) used the high-resolution two-dimensional gel electrophoresis system of O'Farrell (1975) to analyse the proteins synthesised by seven established cultures of P. falciparum. A considerable amount of electrophoretic variation was revealed by this approach. Out of 35 major proteins analysed, 14 varied electrophoretically among seven isolates screened. For each of

these variable proteins two to six different variants were detected among these isolates. This high degree of protein variation allowed each isolate to be distinguished from the others by the combination of electrophoretic variants which it exhibited. Thus the results show that this technique is a very powerful one for characterising parasite isolates. As only seven isolates - two from W. Africa and five from S.E. Asia - were compared, little can be said about geographical variation within the species. As found with antigen and enzyme typing, the same variants of some polypeptides were found in both groups of isolates and several differences were seen amongst isolates from the same area. However, certain variants of four polypeptides were found exclusively among the African strains and these results suggest that geographically restricted variants may exist.

1.6 Aims of this Thesis

The general objectives of this thesis are twofold. The first is to extend the two-dimensional gel analysis of protein variation in P. falciparum initiated by Tait (1981). The second is to use the same technique to systematically identify and characterise the proteins of this parasite. The studies on intraspecific variation were directed towards specific aspects of the population structure of P. falciparum. It was hoped to determine whether the parasites of this species form a single interbreeding population or a series of non-interbreeding groups and to investigate whether some degree of sub-speciation or regional variation exists between or within parasites from different

continents. In addition, it was of interest to examine the heterogeneity which may exist among the parasites isolated from a single infected individual. As outlined in Section 1.2, all these questions have important implications for the biology, epidemiology and control of malaria.

The proteins of P. falciparum resolved by two-dimensional gel electrophoresis have been characterised in several ways. Work was carried out to study the stage-specific synthesis of proteins during the erythrocytic cycle, to identify proteins which are glycosylated and to analyse parasite antigens. The results of these studies may have some bearing on the interpretation and molecular basis of the variation observed in the proteins of different parasite isolates. Such information is also of value in its own right. By identifying and cataloguing a large number of parasite products and then by collating information about their biochemical and biological properties it will be possible to build up a bank of data about them which may be of value in future research.

ABBREVIATIONS USED :

CPK	Creatine phosphokinase
DMSO	Dimethylsulphoxide
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene bis(oxyethylenenitrilo)-tetraacetic acid
IEF	Isoelectric focusing
MW	Molecular weight
NP40	Nonidet-P40
PAGE	Polyacrylamide gel electrophoresis
pI	Isoelectric point
PMSF	Phenylmethylsulphonylfluoride
POPOP	1,4-di-2-(5-phenyloxazolyl)-benzene
PPO	2,5-diphenyloxazole
SDS	Sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethylethylenediamine
TCA	Trichloroacetic acid
TRIS	Tris(hydroxymethyl)aminomethane
1DGE	One-dimensional gel electrophoresis
2DGE	Two-dimensional gel electrophoresis

E. and S. E. Scotland Regional Blood Transfusion Service. Cultures were maintained in group A or O blood cells at approximately 5% haematocrit in RPMI 1640 medium (Gibco) buffered with 25 mM Hepes, 25 mM $NaHCO_3$ and supplemented with 10% human serum (A, O or AB depending on blood cell type) and 10µg/ml gentamycin sulphate. The culture medium was normally replaced at 24 hour intervals and parasites were subcultured every third or fourth day by dilution with uninfected blood cells (washed in RPMI/Hepes/$NaHCO_3$) to a parasitaemia of approximately 0.5%. The parasites multiplied 10-16 fold over the following 4 day period. Growth was monitored by fixing blood smears in methanol and then staining for 20 minutes in a 10% (v/v) solution of Giemsa stain in Sorenson's buffer (pH 7.2).

Stringent sterile technique was required to prevent bacterial or fungal contamination of cultures as well as contamination of one P. falciparum isolate with parasites of another. Contaminating micro-organisms occurred only rarely and it was found that bacteria could usually be cleared by incubating the culture for 24 hours in medium containing 5 µg/ml chloramphenicol, 50 µg/ml neomycin and 60 µg/ml penicillin G. In order to check that cross-contamination between isolates was not occurring, a dish of uninfected blood cells was maintained in parallel with infected cultures and was monitored over a period of weeks for the presence of parasites. No parasites appeared in the control dish providing that good sterile technique was practised.

2.1.3 Cryopreservation of parasites

Many of the isolates used were recovered from cryopreserved material and samples of all the isolates cultured were returned to liquid nitrogen storage for future use. Samples were prepared for cryopreservation by the method of Rowe et al (1968). Parasitised cells were resuspended in an equal volume of a 28% glycerol, 3% sorbitol and 0.65% NaCl solution and then frozen by immersion in liquid nitrogen.

Isolates were routinely recovered by the method of Rowe et al (1968). The cells were thawed rapidly at 37°C, resuspended in 3.5% NaCl, washed with culture medium, diluted with washed, uninfected blood and returned to routine culture. When the method of freezing was not known or when previous attempts using the Rowe method failed then a sorbitol based procedure was used instead. Thawed cells were kept on ice and 1 ml of a cold 17.5% solution of sorbitol in RPMI/Hepes/$NaHCO_3$ was added dropwise with shaking. The suspension was then centrifuged and the process was repeated with 10%, 7.5%, 5% and 2.5% sorbitol solutions and finally without sorbitol prior to the addition of washed, uninfected cells and culture medium. This method was often more successful than the Rowe method. In both cases it was advantageous to change the culture medium twice daily for the first week or so and to add small amounts of washed blood more frequently than for routine culturing. However, several attempts to recover cryopreserved patient's blood failed due to the induction of gametocytogenesis and the loss of the

asexual parasites. The use of lower haematocrits or higher serum concentrations had little effect on the viability of such samples. Difficulties were also experienced with some established cultures which had been cryopreserved in other laboratories. Problems in recovering parasites limited to some extent the number of isolates, particularly those of Gambian origin, analysed in this thesis.

2.1.4 Synchronisation of cultures

Methods for synchronising the normally asynchronous cultures were based on two procedures: i) the selective lysis of trophozoite- and schizont-infected erythrocytes by sorbitol (Lambros and Vanderberg, 1979) and ii) the concentration of schizont-infected cells by flotation in modified gelatin solutions (Jensen, 1978; Pasvol et al, 1978). Synchronisation was achieved either by using sorbitol treatments only or by combining the two methods. A single treatment with sorbitol, as detailed below, produces a culture of predominantly young parasites. Subsequent, carefully timed sorbitol treatments are then used to kill the older parasites and narrow the age-range of the surviving parasites. This method of synchronisation can be used on all isolates. The alternative method is to concentrate schizont-infected cells with Plasmagel, incubate them with uninfected erythrocytes, allow reinvasion to take place and then use the sorbitol method to kill remaining schizonts and thus produce a culture of young rings of a known age-range. Although this procedure gives good synchrony in a single step it has two disadvantages. One is

that the yield of schizonts after flotation in Plasmagel is low and, therefore, a large volume of the starting culture is needed. The second is that it is not applicable to all isolates since the schizont-infected cells of some strains sediment in Plasmagel solutions.

Synchronisation by sorbitol treatments (based on Myler et al, 1982). An asynchronous starting culture of $\geq$5% parasitaemia was centrifuged (1200 g, 5 mins) and the cells resuspended in 4 vols. of 5% sorbitol. After incubating for 5 mins. at room temperature the suspension was centrifuged, fresh medium was added to the cells and they were cultured as normal. This procedure was repeated after 24 hours and uninfected blood was added to give 1-4% parasitaemia. Growth was monitored and the sorbitol treatment repeated during the period of reinvasion and/or 24 hours later over 3 or 4 successive growth cycles until the required parasitaemia and degree of synchrony was achieved.

Synchronisation by sorbitol/Plasmagel treatments (based on Kilejian, 1980a). An asynchronous culture was treated once with sorbitol as above and cultured until the parasites were late trophozoites and schizonts. The culture was then centrifuged (1200 g. 5 mins), the cells resuspended in culture medium to a 40% haematocrit, mixed with an equal volume of Plasmagel (3% (w/v) modified gelatin in physiological saline) and incubated at 37^{o}C, 30 mins. The 'supernatant' containing mainly mature schizonts was collected, supplemented with washed, uninfected blood and medium and returned to culture. The culture medium was replaced once or twice over the

next few hours to remove the Plasmagel. (Centrifugation was not used to wash the cells because the Plasmagel treatment left the parasitised cells very susceptible to lysis by physical or chemical means). Growth was monitored and the cultures were treated with sorbitol at an arbitrary time after the start of reinvasion to kill the remaining schizonts and produce a culture of young rings. If necessary, the Plasmagel/sorbitol treatment was repeated to improve synchrony during later cycles.

2.1.5 Preparation of free parasites by saponin-lysis

Parasites were partially purified from cultures by using saponin to selectively lyse the erythrocytes followed by centrifugation to separate the red cell ghosts and soluble components from the parasites. Although this removes most of the erythrocytic material, some red cell membrane may still be associated with the parasite preparation (Kreier, 1977). The method used involved cooling the culture on ice, adding 0.5 volumes of a 0.15% (w/v) solution of saponin in RPMI/Hepes/$NaHCO_3$, mixing and leaving on ice until suspension turned clear (5-10 mins). 3 vols. of RPMI/Hepes/$NaHCO_3$ were then added and the suspension was centrifuged at 2500 g, 20 mins. The supernatant and layer of red cell ghosts were discarded and the parasite pellet washed x 2 in appropriate medium.

2.2 Radioisotopic Labelling of Proteins and Glycoproteins

Labelled precursors were incorporated biosynthetically into

P. falciparum proteins by incubating either saponin-lysed parasite preps. or parasitised erythrocytes with labelled amino acids or glucosamine (for glycoproteins). The incubation medium used for metabolic labelling was varied depending on the type of parasite preparation and the nature of the labelled precursor used. The protocols and media used are detailed below.

2.2.1 Labelling saponin-lysed parasite preparations (Tait, 1981)

Parasites were prepared as detailed in Section 2.1.5 and washed two or three times in methionine-free incorporation medium (a buffered salt solution containing glucose, amino acids, vitamins and 10% foetal calf serum as detailed by Tait, 1981) or in methionine-free RPMI. Approximately 2-4 x 10^8 parasites ($\equiv$ 25 µl packed cells) were resuspended in 100 µl either of the medium used for washing or of a physiological buffered salt solution (Tait, 1981). 25-55 µCi of ^{35}S-methionine (920 Ci/mmol, Amersham International) or 25-75 µCi of ^{3}H-methionine (15 or 87 Ci/mmol, Amersham) were added and the suspension was incubated at 37°C for 60-120 mins, with occasional shaking. Incorporation was terminated by adding an equal volume of O'Farrell lysis buffer (see Section 2.3.2), mixing until clear and then freeze - thawing x 2 to lyse cells.

2.2.2 Labelling parasitised erythrocytes

Cultures for labelling were grown to parasitaemias of 3-5% (1.5 - 3 x 10^7 parasites/ml) and the culture medium was replaced with an equal volume of the appropriate incubation medium and

labelled precursor as listed below:-

i) ^{3}H- or ^{35}S-methionine. 13-33 μCi/ml ^{35}S-methionine (920 Ci/mmol, Amersham) or 17-67 μCi/ml ^{3}H-methionine (87 Ci/mmol, Amersham) was added to methionine-free medium made up to the same composition as RPMI 1640 and supplemented with Hepes, $NaHCO_3$, gentamycin and 10% human serum as for complete culture medium.

ii) ^{3}H- or ^{14}C - protein hydrolysate. 17-37 μCi/ml of a ^{14}C - protein hydrolysate (>50 mCi/milliatom C, Amersham) or 33-67 μCi/ml of an equivalent ^{3}H-amino acid mixture (0.2-60 Ci/mmol, Amersham) was added to an amino acid-free incubation medium prepared as in i).

iii) ^{3}H- or ^{14}C - amino acid mixture. 50 μCi/ml of a high specific activity mixture of ^{3}H-leucine, lysine, phenylalanine proline and tyrosine (70-170 Ci/mmol of each amino-acid, Amersham) or 13 μCi/ml of the equivalent mixture of ^{14}C - amino acids (290 - 520 mCi/mmol, Amersham) was added to leucine, lysine, proline, phenylalanine and tyrosine-free medium as above.

iv) ^{3}H-glucosamine (22.6 Ci/mmol, Amersham) for labelling parasite glycoproteins was added at 50 μCi/ml to complete RPMI culture medium.

Cultures were incubated with labelled medium at 37°C in candle jars for 8-26 hours. Parasites were then harvested by saponin lysis (Section 2.1.5) and solubilised as described in Section 2.3. A quantitative and qualitative comparison of the different

labelling precursors and regimes is given in Chapter 3.

2.3 Preparation of Samples for Gel Electrophoresis

2.3.1 Solubilisation for one-dimensional (1D) SDS gels

Parasite pellets were suspended in SDS sample buffer (42 mM Tris, pH 6.8, 1% (w/v) SDS, 5% (v/v) β - mercaptoethanol, 9% (v/v) glycerol, 0.002% (w/v) bromophenol blue), heated at 95°C for 2 mins., cooled and then centrifuged at 10,000 g for 2 mins in a micro-centrifuge to remove any insoluble material. Molecular weight marker proteins were solubilised as above at concentrations of 1-1.5 mg/ml. To solubilise myosin it was necessary to include 8 M urea and to increase the heating time. To solubilise immune precipitates (section 2.8) the concentrations of SDS and urea were increased to 4% and 6 M respectively.

2.3.2 Solubilisation for two-dimensional (2D) gels

Parasite pellets were resuspended in O'Farrell lysis buffer (9.5 M urea, 2% (v/v) Nonidet -P40 (NP40), 2% (v/v) ampholines, 5% (v/v) β -mercaptoethanol) at 0.5 - 1 x 10^7 parasites/10 µl, mixed until clear and then subjected to 2 or 3 cycles of freeze-thawing to lyse the cells. Samples were centrifuged at 10,000 g for 2 mins. to remove insoluble debris and the supernatants were either used immediately or stored at -20° or -70°C. In order to fully solubilise double-immune precipitates it was necessary to solubilise initially with SDS by resuspending the precipitate in 2% (w/v) SDS in 10 mM

Tris, pH 7.6 and heating at 95°C, 5 mins. After cooling, urea, ampholines and β-mercaptoethanol were added to the final concentrations given above and NP40 to a final concentration of 8 %.

2.3.3 Scintillation counting

Samples of labelled preparations were spotted onto glass fibre discs (Whatman GF/A) and allowed to dry. The filters were then processed by the method of Bollom (1966) which involves sequential washing in cold and hot trichloroacetic acid (TCA) solutions to remove nucleic acids and amino acids followed by ethanol/ether washing to remove lipids. Thus, only proteins should be left on the filters which were immersed in a PPO/POPOP toluene-based scintillation fluid and counted in a Nuclear Chicago scintillation counter.

2.3.4 Sample Loading

For ID gel electrophoresis approximately 4-5 x 10^4 cpm of ^{35}S-methionine-labelled extracts (roughly equivalent to 1-2 x 10^6 parasites) or 1-6 x 10^4 cpm of ^{3}H-glucosamine-labelled extracts (1-4 x 10^7 parasites) were loaded into each sample well.

For 2D gel analysis 1-5 x 10^5 cpm of ^{35}S-methionine-labelled samples (0.4 - 5 x 10^7 parasites) or 1-5 x 10^4 cpm of ^{3}H-glucosamine-labelled samples (1.5-4 x 10^7 parasites) were run on each gel. For double-label autoradiography (section 3.3) 1.5-3 x 10^5 cpm of a ^{14}C-amino acid labelled extract was co-electrophoresed with 1-2 x 10^6 cpm of a ^{3}H-labelled extract so that the ratio of ^{14}C : ^{3}H cpm was 1 : 7 (equivalent to a total of 3-9 x 10^7 parasites).

2D gels which were analysed by silver staining were loaded with $2\text{-}5 \times 10^7$ parasites per gel.

2.4 Polyacrylamide Gel Electrophoresis (PAGE)

2.4.1 One-dimensional (1D) SDS PAGE

This was performed basically as described by Laemmli (1970) using a discontinuous buffer system. A Hoeffer vertical slab gel apparatus (SE 600) was used.

The gel dimensions were: thickness, 1.5 mm; width, 15 cm; resolving gel length, 12 cm; stacking gel length, 1.5-3 cm. All glass plates were washed in mild detergent, rinsed, air-dried and then cleaned with ethanol immediately before use.

The ratio of acrylamide : methylene bis acrylamide used was 29.2 : 0.8. The resolving gel was made up to the following composition: 0.375 M Tris-HCl pH 8.8, 10% acrylamide (w/v), 0.1% (w/v) SDS, 0.032% (w/v) ammonium persulphate, 0.0002 (v/v) TEMED. The stacking gel contained: 0.11 M Tris-HCl, pH 6.8, 4.3% acrylamide, 0.1% SDS, 0.036 % ammonium persulphate, 0.0005 % TEMED. The electrode buffer was 25 m.M Tris, 0.191 M glycine and 0.1% SDS.

The Tris/acrylamide resolving gel solution was degassed by evacuating for 2-5 mins. SDS was added and polymerisation initiated by adding stock solutions of ammonium persulphate and TEMED. After pouring, gels were overlaid with 0.1% SDS solution to a depth of ~3 mm and then allowed to set (30-60 mins). Once

set the gel surface was rinsed with distilled water. The stacking gel was prepared as described above. A 10-slot well former was positioned in the stacking gel solution and setting occurred within 15-30 mins. After removing the well former, the wells were rinsed with distilled water and then electrode buffer. Samples, prepared as detailed in section 2.3 were loaded into the wells by a Pipetman micropipette. Unused wells were loaded with an equivalent volume of tracking solution (60% (v/v) glycerol, 26% (v/v) β - mercaptoethanol, 0.007% (w/v) bromophenol blue). All samples were then gently overlaid with electrode buffer. The electrophoresis apparatus was then assembled, the buffer tanks filled with electrode buffer and air bubbles removed from the lower surface of the resolving gel. Electrophoresis was carried out at constant current (12-15 mA per gel) for 16-20 hours until the bromophenol blue tracking dye was approximately 1 cm from the bottom of the resolving gel. Gels were then removed from the apparatus and processed as detailed in Sections 2.5 and 2.6.

2.4.2 Two-dimensional gel electrophoresis (2DGE)

This was performed as described by O'Farrell (1975). Isoelectric focusing (IEF) was done in a Hoeffer multiple tube gel electrophoresis unit. The gel tubes were cleaned before use by soaking in chromic acid for 24 hours, rinsing with water, soaking in IM NaOH for a further 24 hours, re-rinsing and then finally washing with ethanol. The gels were 3 mm in diameter and 12 cm

in length. The composition of the gels was as follows: 9.1 M urea, 4% acrylamide (acrylamide : methylene bis acrylamide = 28.4 : 1.6), 2% (v/v) NP40, 1.6% (w/v) 5 - 7 ampholines, 0.4% (w/v) 3.5 - 10 ampholines. 0.01 M H_3PO_4 was used as the anode solution and degassed 0.02 M NaOH as the cathode solution.

The gel solution was prepared as above, degassed and polymerisation was initiated by the addition of ammonium persulphate and TEMED to final concentrations of 0.01% (w/v) and 0.0007% (v/v) respectively. The gel solution was pipetted into the glass tubes (which had been sealed at one end with parafilm), and was then overlaid with 8 M urea. The gels were left to polymerise for 1-2 hours. This and all subsequent steps were performed in a 25°C constant temperature room to prevent the urea from crystallising. After setting, the overlay was removed and replaced with 20 µl of lysis buffer (see section 2.3.2) which was in turn overlaid with distilled water. The gels were left for a further 1-2 hours. The overlay was replaced with a further 20 µl of lysis buffer followed by degassed cathode solution. The apparatus was assembled for electrophoresis and the gels were pre-run at 200 V constant voltage for 15 mins, 300 V for 30 mins and finally at 400 V for 30 mins. Following this, the cathode electrode solution and the gel overlays were discarded. The samples, prepared as described in section 2.3, were loaded onto the gels and overlaid with 10 µl of a solution of 2% ampholines in either 4 or 7 M urea followed by freshly degassed cathode electrode solution. Electrophoresis was performed at 400 V

for 16-17 hours i.e. 6,400 - 6,800 V. hours. The gels were removed from the tubes either by using a syringe and needle to free the gel by forcing water between the gel and the sides of the tube or by connecting the tube to a syringe via a length of tubing and then slowly pushing the gel out of the tube. Each gel was then placed in 5 ml of equilibration buffer (10% (v/v) glycerol, 5% (v/v) β - mercaptoethanol, 2.3% (w/v) SDS, 0.125 M Tris - HCl. pH 6.8) and shaken for 30-60 mins. Gels were then either loaded onto the second dimension gel or were frozen and stored at -20°C or -70°C.

SDS PAGE. The second dimension gels were prepared, poured and electrophoresed as described for the 1D SDS gels but with some modifications. 1.5 mm thick slab gels were used for electrophoresis of immunoprecipitates, protein extracts for silver staining and some ^{35}S-methionine labelled extracts. All other 2D gel samples were run on 0.75 mm thick slab gels. The stacking gels were 2-4 cm long and instead of making wells, a level surface was formed 2-5 mm below the top of the gel plates by overlaying with 0.1% SDS as for the running gels. After rinsing the surface of the polymerised stacking gel, the gel apparatus was assembled and the isoelectric focusing tube gel was loaded as follows: hot 1% (w/v) agarose in equilibration buffer was pipetted into the gap between the top of the gel and the gel plates, the tube gel was immediately positioned along the top edge of the plates and sealed in place with an even covering of agarose and left to solidify for 1-2 mins. The top tank was then filled with

electrode buffer plus a few drops of 0.1% (w/v) bromophenol blue tracking dye.

2.5 Protein Staining Methods

Two methods of staining gels for protein were used depending on the sensitivity required. Coomassie Blue was used to visualise molecular weight and pI marker proteins and a more sensitive silver staining technique was used to detect parasite proteins.

2.5.1 Coomassie Blue staining

Two-dimensional gels were routinely stained by immersing in 0.1% Coomassie Blue R, 50% trichloroacetic acid for 30-60 mins., and then destaining in several changes of 7% acetic acid. However, in order to visualise some major parasite proteins in addition to marker proteins, the following more sensitive procedure was used. Gels were fixed and stained by shaking them in 0.25% Coomassie Blue R, 50% methanol and 7.5% acetic acid for 1-2 hours. Destaining was initiated by soaking the gels in 50% methanol, 7.5% acetic acid for 1 hour and then continued in several changes of 5% methanol, 7.5% acetic acid.

2.5.2 Silver staining

The ultrasensitive colour-staining method of Sammons *et al* (1981) was used as detailed below. Gels were fixed by shaking in 2 changes of 50% ethanol, 10% acetic acid for 1 hour each. 5.5 gel volumes of the fixing solution was used in each case. This was

repeated using 25% ethanol, 10% acetic acid and then 10% ethanol, 0.5% acetic acid. Gels were then incubated in 3 volumes of degassed $AgNO_3$ (1.9 g/l) for 2 hours. After rinsing briefly in degassed, distilled water, the gels were immersed for 10 mins. in 5.5 volumes of reducer (degassed 87.5 mg/l $NaBH_4$ in 0.75 M NaOH to which 7.5 ml/l formaldehyde was added immediately before use). Finally, gels were soaked in 2 changes of 7.5 g/l Na_2CO_3 for 1 hour each.

A major problem with this method was that both 1D and 2D SDS gels showed intensely stained horizontal bands at positions corresponding to molecular weights (MW) of 60 and 66 kd which obscured much detail in these parts of the gel. Filtering all stock gel solutions, buffers and staining solutions through 0.22 um millipore filters did not remove these bands and they appeared even when no protein was run on the gels. However, as suggested by Guevera et al (1982), their presence did correlate with that of β-mercaptoethanol. When this was omitted from the 1D SDS gel samples or from the 2D gel equilibration buffer then the bands disappeared or were substantially reduced in intensity. Unfortunately the omission of β-mercaptoethanol altered the positions of some proteins on 2D gels and replacing mercaptoethanol with dithiothreitol resulted in reappearance of the stained bands. In the light of these observations it is probable that the contaminating bands were not proteins but were artefacts produced by chemical interactions between a reducing agent (mercaptoethanol or dithiothreitol in this case) and another sample or gel reagent.

This artefactual staining seems to be a characteristic of current high-sensitivity silver staining techniques. Published photographs illustrating the method used here (Sammons *et al*, 1981) and also that of Porro *et al* (1982) show unexplained stained bands at approximately the same MW as noted above. These were also seen, although less intensely, on gels of *P. falciparum* proteins stained by the less sensitive silver staining method of Morrissey (1981).

2.6 Autoradiography

Fluorography was carried out either following staining with Coomassie Blue or after fixing the gels in 50% TCA for 30 mins. The method used was that of Laskey and Mills (1975). Gels were shaken in 3 changes of DMSO for 30 mins. per wash and then shaken in a 22.2% (w/v) solution of PPO in DMSO for 3 hours. Gels were then washed for at least one hour in several changes of distilled H_2O before being dried and exposed to X-ray film as described below.

Gel drying. Gels were dried down onto sheets of Whatman 3 MM paper in an LKB gel drier. 1.5 mm thick gels were dried for 70 minutes and 0.75 mm gels for 40 minutes.

Autoradiography. Fluorograms were made by exposing PPO-treated gels to Kodak X-omat RP film. The film was preflashed before exposure as detailed by Laskey and Mills (1975). The dried gel and film were clamped between glass plates, wrapped in black plastic bags and stored at -70°C. Direct autoradiographs were prepared in the same way but Kodak No-Screen X-ray film was

used and the exposures were made at room temperature.

2.7 Calibration of One- and Two- Dimensional Gels

2.7.1 Molecular weight calibration

The following proteins were used as standards in order to estimate the molecular weight of the parasite proteins resolved on SDS gels: myosin (205 kd), β-galactosidase (116 kd), phosphorylase b (97 kd), bovine serum albumin (68 kd), ovalbumin (43 kd) and trypsin (23.3 kd). Each was solubilised as described in Section 2.3.1. 4-40 µg of each protein was mixed for visualisation by Coomassie Blue staining and 0.4 - 4 µg for silver staining. For use on 1D gels the mixed sample was loaded directly into one of the wells. For 2D gels, the sample was mixed with a small volume of hot 1% agarose in equilibration buffer, allowed to set in a glass tube and then extruded and embedded alongside the isoelectric focusing gel on top of the SDS slab gel.

2.7.2 Isoelectric point (pI) calibration

The isoelectric focusing gels were calibrated by two different methods.

i) Direct pH measurements. Immediately after electrophoresis gels were sliced into 5 mm segments. Each was shaken in 1 ml of degassed 25 mM KCl for 1-2 hours and the pH measured directly using a pH electrode. The results were then used to plot the pH profile created during isoelectric focusing (see fig. 2.1). Proteins

focused on parallel gels could then be assigned pI values. However it should be noted that even within a single isoelectric focusing gel run slight differences in pH profile can occur (especially at the ends of the gels) due to small variations in gel length or volumes of sample loaded. The main disadvantage of this method is that the level of accuracy attainable is not very high. Thus the pI values determined may not be precise enough to define proteins unambiguously when making comparisons between different gel runs or gels produced by other laboratories. Anderson and Hickman (1979) estimated direct pH measurement to be reproducible only to within 0.1 pH unit between different laboratories as compared with differences of about 0.01 pH unit produced by single charge changes in many proteins.

ii) Internal calibration. Carbamylation trains of rabbit muscle creatine phosphokinase (CPK) were used to internally calibrate gels by the method of Anderson and Hickman (1979). A 5 mg/ml solution of CPK in 8 m urea was prepared and samples were heated at 95°C for 0, 2, 4, 6, 8, 10, 15, 30 and 60 minutes. Longer heating in urea produced greater degrees of carbamylation and hence proteins of more acidic pI. Equal volumes of each sample were pooled, run on 2D gels and stained with Coomassie blue to examine the train of spots produced. The proportions of each sample in the stock mixture could then be adjusted to give a reasonably uniform carbamylation train. This train of spots covered about three quarters of the focusing gel leaving the acidic end uncharted. Following the precedent of Anderson and Hickman, the most basic, unmodified

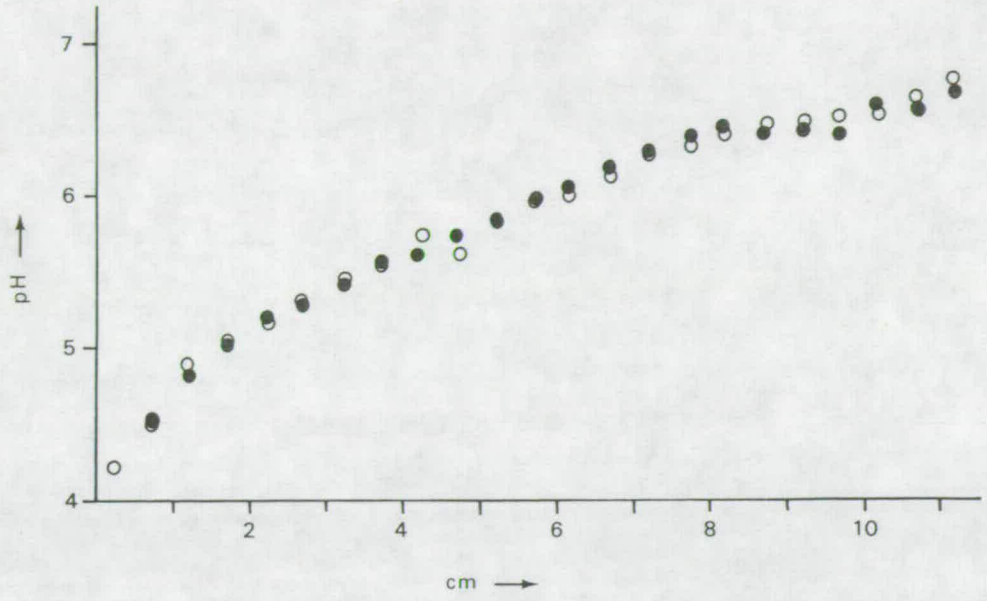


Fig. 2.1 pH gradient established during isoelectric focusing. Duplicate gels were analysed as detailed in Section 2.7.2

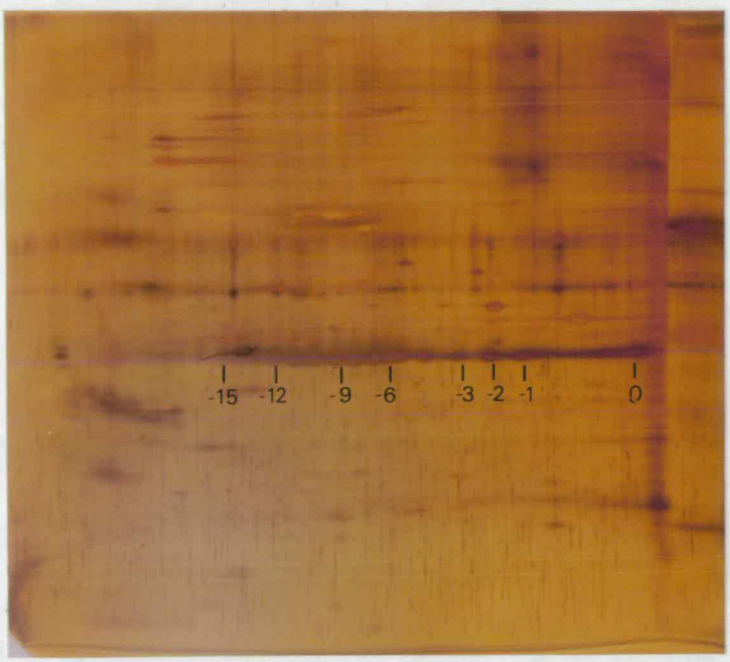


Fig. 2.2 Co-electrophoresis of parasite proteins and carbamylated creatine phosphokinase. Proteins were visualised by silver staining and charge isomers in the carbamylation train are numbered (-1, -2 etc.) relative to unmodified creatine phosphokinase (0).

CPK spot was numbered 0 and the successively more acidic members of the charge train were numbered -1, -2 etc. In theory, at least 30 spots should be present but in practice only about 20 could be resolved and identified unambiguously (fig. 2.2).

To internally calibrate gels, 5 µg (for silver staining) or 50 µg (for Coomassie Blue staining) of carbamylated CPK was mixed with a sample of P.falciparum proteins, subjected to 2D gel electrophoresis and then stained for protein. Silver staining was particularly useful for calibration because it allowed all the major parasite proteins and the CPK charge train to be visualised on the same gel as illustrated in fig. 2.2. Thus the parasite proteins can be characterised by their position relative to members of the CPK train.

This method of calibration avoids the problems inherent in direct pH measurements and allows more precise comparisons to be made between different gels. However, it does not give pI values directly and at present these can only be estimated by using direct pH measurements such as the one described above.

The combined use of all the above methods has made it possible to characterise the major P. falciparum proteins in terms of approximate pI, position relative to members of the CPK carbamylation train and molecular weight. Having been characterised, these parasite proteins were then themselves utilised as internal standards on other stained gels and autoradiographs.

2.8 Immunochemical Techniques

2.8.1 Anti- P. falciparum sera

A freeze-dried preparation of human serum known to react with falciparum malaria was obtained from a donor in northern Thailand (Changmai) who had been infected with malaria several times. Rabbit serum was raised against saponin-lysed parasites of isolate SK17 in this laboratory as described below. In addition, a further sample of rabbit serum raised against isolate K1 was provided by Dr. R. Hall (Dept. of Molecular Biology, Edinburgh University).

2.8.2 Preparation of rabbit antisera

i) against P. falciparum. Parasites of isolate SK17 were harvested by saponin lysis of a partly synchronised culture. Harvesting was done after reinvasion had started, most of the parasites then being mature schizonts. Parasites were resuspended in 0.85% NaCl at an approximate density of 1×10^9 parasites/ml. 1 ml of suspension was emulsified (by repeatedly forcing through a 26 G $^3/_8$ syringe needle) with an equal volume of 9:1 (v:v) Drakeol 6 VR: Arlacel A. A double emulsion was then made with 2 volumes of 2% (v/v) Tween 80 in 0.85% NaCl. The rabbit was injected with this preparation subcutaneously at several positions around the back of the neck. A blood sample was taken from a marginal ear vein for use as an unimmunised control. A second injection of antigen was given 4 months later. The parasites were prepared from an

asynchronous culture and approx. 2×10^8 parasites were injected. 10 ml blood samples were taken after a further 8 and 22 days. To prepare serum the blood was left at room temperature for 1 hour or until clotting had started. The clot was rimmed by inserting a pasteur pipette between the clotting blood and the sides of the collecting bottle and then left at 4°C overnight. Serum was removed two or three times as the clot contracted. This was pooled, centrifuged at 2000 g for 15 mins and the supernatant was aliquoted and stored at -20°C or -70°C.

ii) against human IgG. IgG was purchased from Sigma and 100 µg was emulsified in Freunds complete adjuvant before injection subcutaneously into each of two rabbits. After 28 days a further 100 µg IgG in phosphate-buffered saline was injected intravenously. 14 days later the rabbits were bled and serum prepared as described above. Bleeding was repeated at 1-2 week intervals.

2.8.3 Preparation of parasite extracts for immunochemical analysis

Cultures were labelled with ^{35}S-methionine as described in section 2.2. Extracts were prepared either from saponin-lysed parasite preparations or from intact parasitised erythrocytes. In the former case, the parasites were solubilised by resuspending in 0.5% NP40, 0.15 M NaCl, 5 mM EDTA, 0.02% azide, 50 mM Tris-HCl, pH 7.4 at 1-4 x 10^8 parasites/ml. After incubation at 4°C, 30 mins. insoluble material was removed by centrifuging at 10,000 g, 30 mins. and the soluble extract was stored at -70°C in

aliquots of 2-5 x 10^5 cpm. Alternatively, infected erythrocytes were washed twice in RPMI/Hepes/$NaHCO_3$ and then resuspended in 1% NP40, 0.9% NaCl, 1mM EDTA, 1mM EGTA, 2mM PMSF, 10 mM phosphate buffer, pH 7.4 at 1-1.5 x 10^8 parasitised cells/ml. This was extracted for 1 hour at 4°C with occasional shaking, centrifuged at 10,000 g, 30 mins. and aliquoted as above.

2.8.4 Immune precipitation

Two methods of immunoprecipitating P. falciparum proteins were used. In the first soluble immune complexes were isolated by adsorption on Protein A - sepharose. In the second rabbit anti-human IgG serum was used to precipitate human antibody-parasite protein complexes directly.

i) Immunoprecipitation with Protein A-sepharose (based on Kessler (1975, 1976)). A suspension of Protein A-sepharose (Sigma) was extensively washed in 0.5% NP40 in NET buffer (0.15 MNaCl, 5 mM EDTA, 0.02% azide, 50 mM Tris-HCl, pH 7.4) and resuspended at 30 mg/ml. The labelled parasite extract was pre-absorbed by incubating with Protein A-sepharose (100 µl extract per 0.5 ml Protein A-sepharose) at 25°C, 30 mins. The Protein A-sepharose was removed by centrifuging at 10,000 g, 2 mins. and the preabsorbed extract was mixed with 30 µl of test serum (previously dialysed against 0.05% NP40 in NET buffer) for 30 mins., 25°C. 0.5 ml of Protein A-sepharose suspension was added and the mixture left at 25°C, 20 mins with occasional shaking.

The Protein A-sepharose was then washed four times by pelleting and resuspending in 0.05% NP40 in NET buffer. Immune complexes were eluted either by a) incubating the Protein A-sepharose with 75 µl of 0.2 M citrate, pH 5.0 at 25°C, 30 mins or b) heating in 75 µl of 4% SDS in 6 M urea at 95°C, 2 mins. After removing the Protein A-sepharose by centrifugation for 2 mins in a microcentrifuge the eluate was prepared for gel electrophoresis as described in section 2.3.

ii) Double immune precipitation. Prior to immunoprecipitation it was necessary to determine the ratio of rabbit antiserum : human serum needed to give the maximum precipitation of human IgG. This was estimated empirically by mixing the sera in different proportions, incubating at 37°C, 30 mins. and 4°C, overnight and then measuring the amount of precipitate formed for each ratio.

Labelled protein extracts were diluted with PSEEP buffer (10 mM phosphate buffer, pH 7.4, 0.9% NaCl, 1mM EDTA, 1 mM EGTA, 2 mM PMSF) to reduce the NP40 concentration to 0.2%. 10 µl of immune or control human serum was added and the mixture incubated at 37°C for 30 mins. The NP40 concentration was re-adjusted to 0.5% and incubation was continued at room temperature for 10 mins. The appropriate amount of rabbit anti-human IgG serum was then added and the solution was left at 37°C, 30 mins. and at 4°C, overnight. The precipitate was collected, washed three times in 0.5% NP40 in PSEEP buffer and then solubilised for 1D or 2D gel electrophoresis as described in section 2.3.

Proteins bound by monoclonal antibodies 2.2 and 7.3

Dr. R. Hall (Dept. of Molecular Biology, Edinburgh University) chemically linked these antibodies to CNBr-activated sepharose and used the products for affinity chromatography by passing ^{35}S-methionine labelled extracts of isolate K1 through the affinity columns. Small samples of the antibody-sepharose with bound parasite protein were provided by Dr. Hall. These were mixed with O'Farrell lysis buffer, incubated at room temperature for 30 mins. to dissociate the proteins and then centrifuged to remove the sepharose-bound antibody. The proteins eluted were then analysed by 2D gel electrophoresis.

BIOSYNTHETIC LABELLING AND 2D GEL ELECTROPHORESIS OF P. FALCIPARUM PROTEINS

3.1 Introduction

Radioisotopic labelling is a convenient way of identifying the proteins synthesised by P. falciparum parasites. As mature erythrocytes do not synthesise proteins, biosynthetic labelling overcomes the restraints imposed by the relatively small amounts of impure parasite material obtainable from in vitro cultures. Tait (1981) developed a procedure for incubating saponin-lysed parasite preparations with ^{35}S-methionine. Other workers have labelled whole cultures rather than free parasites (e.g. Brown et al, 1982b; Myler et al, 1982) and some have used labelled precursors other than ^{35}S-methionine (e.g. Kilejian, 1980a, 1980b).

The biochemical analysis of labelled P. falciparum proteins has previously depended to a large extent upon 1-dimensional SDS acrylamide gel electrophoresis. As a result, protein characterisation has suffered from the difficulty of distinguishing unambiguously between proteins of the same or similar molecular weight. The development of 2-dimensional gel electrophoresis (2DGE) which separates proteins first by charge and then by molecular weight has produced a system of very high resolving power (O'Farrell, 1975). The application of this technique has successfully allowed the dissection and analysis of complex protein mixtures (e.g. O'Farrell, Leigh 1975; Brown and Langley, 1979; Walton, Styer and Gruenstein, 1979). P. falciparum proteins have been separated by 2DGE by

Tait (1981), Brown et al (1982b, 1983a, 1983b), Anders et al (1983) and Howard and Reese (1984).

Three requirements for obtaining the maximum amount of information from 2D gels are: i) a sensitive method of protein detection e.g. autoradiography of labelled proteins, ii) standardised methods of labelling, sample preparation and gel electrophoresis to optimise reproducibility between gels and iii) accurate analysis of the resolved proteins. In fact, the major difficulty encountered in using 2DGE is the analysis of the protein patterns produced. When samples are separated on different 2D gel runs minor differences in electrophoretic conditions can result in the protein patterns appearing slightly different. Thus when screening for electrophoretic variation amonst the proteins of different samples, considerable time must be spent carefully comparing the relative positions of particular protein spots on each 2D gel. In practice, this imposes a limit on the number of proteins which can be screened by eye and also on the ability to detect small shifts in protein position. There are two ways round this problem. One is to run several gels of each sample and then to use computer-aided analysis to increase both the number of proteins screened and the precision with which comparisons can be made (Garrels, 1979; Anderson et al, 1981). The other is to incorporate double-label autoradiography into the 2D gel technique.

Double-label autoradiography (McConkey, 1979) allows two different samples to be compared on a single gel. One sample must

be labelled with ^{35}S- or ^{14}C- precursors and the other with the equivalent ^{3}H- precursors. After running both samples on a single two-dimensional gel the isotopes can be differentiated by exposing the gel at different temperatures to two types of X-ray film. Superimposing these two exposures allows the samples to be compared directly after having experienced identical electrophoretic conditions. Thus this technique removes the necessity for assessing the relative positions of all proteins in samples which have been run on separate 2D gels. The advantages of using a double-label method of analysis are that a larger number of proteins can be surveyed visually and that the ease and accuracy of detecting electrophoretic variation is considerably enhanced. The main drawback of the technique is that the autoradiographic exposure times can be lengthy. In order to produce a workable double-label system it is necessary to label proteins to a sufficiently high specific activity so that both the amount of protein applied to each gel and the time required for its detection are minimised. Thus, prior to applying double-label autoradiography to the analysis of parasite proteins on 2D gels it is essential to examine the technical aspects of labelling the proteins and to ensure that the system will allow a large number of proteins to be detected and compared without prohibitively long autoradiographic exposure times.

The aims of the work reported here are:

i) To compare quantitatively and qualitatively the different methods of biosynthetically labelling P. falciparum proteins i.e. labelling

infected erythrocytes or suspensions of saponin-lysed parasites.

ii) To compare the efficiency of incorporation of different labelled precursors into parasite protein and to compare the 2D gel patterns produced.

iii) To develop a suitable labelling system for double-label autoradiography.

iv) To identify and characterise a set of major parasite proteins to provide a framework for subsequent analysis of protein variation in P. falciparum.

3.2 Biosynthetic labelling of P. falciparum proteins

3.2.1 Labelling parasite suspensions

The procedure adopted was based on that of Tait (1981) (Section 2.2). Parasites from asynchronous cultures were freed from erythrocytes by saponin lysis. Parasite suspensions were then incubated in methionine-free medium with either ^{35}S- or ^{3}H-methionine for up to two hours. Protein synthesis was monitored at 30 or 60 minute intervals by removing small volumes of the suspension for scintillation counting (section 2.3). As described by Tait (1981), the rate of incorporation of label into parasite protein was rapid during the first hour of incubation and then declined over the second hour. Extending the length of the incubation period beyond two hours produced only a small increase in the total amount of protein labelled.

Details of the labelling conditions, amount of label incorporated and estimated specific activities of the labelled parasite proteins are

Table 3.1 Incorporation of labelled amino acids by free parasites[i]

Labelled precursor	Specific activity, Ci/mmol	Nos. of exps.	Radioisotope concentration, µCi/100 µl	Parasite density, nos./100 µl	Total protein labelled[ii], ave. dpm/100 µl	Specific activity of labelling, ave. dpm/10^7 parasites
^{35}S-methionine	920	15	25-55	2-5 x 10^8	2.0 x 10^6	1.0 x 10^5
^{3}H-methionine	15	7	25-71	2-5 x 10^8	1.2 x 10^6	0.3 x 10^5
	87	9	38-72	2-5 x 10^8	2.6 x 10^6	0.9 x 10^5

(i) Parasites released from infected erythrocytes by saponin lysis were incubated with labelled methionine for 60-120 minutes (Sections 2.2.1 and 3.2.1).

(ii) TCA-precipitable cpm were detected by scintillation counting (Section 2.2.3) and the dpm incorporated estimated on the basis of a 80% counting efficiency for ^{35}S (or ^{14}C) and 20% for ^{3}H.

shown in Table 3.1. Two specific activities of ^{3}H-methionine were tested (15 + 87 Ci/mmol) and the higher one gave consistently better results in terms of total incorporation and specific activity of labelled protein. Despite the tenfold higher specific activity of ^{35}S-methionine (920 Ci/mmol) the estimated dpm incorporated was approximately the same as that for ^{3}H-methionine (87 Ci/mmol).

A representative 2D gel of a ^{35}S-methionine labelled extract is illustrated in Fig. 3.1 (a). ^{3}H-labelled samples produced identical 2D gel patterns. Approximately 200 proteins can be seen and those which were previously characterised in the study by Tait (1981) are identified in fig. 3.1 (b).

3.2.2 Labelling *P. falciparum* cultures

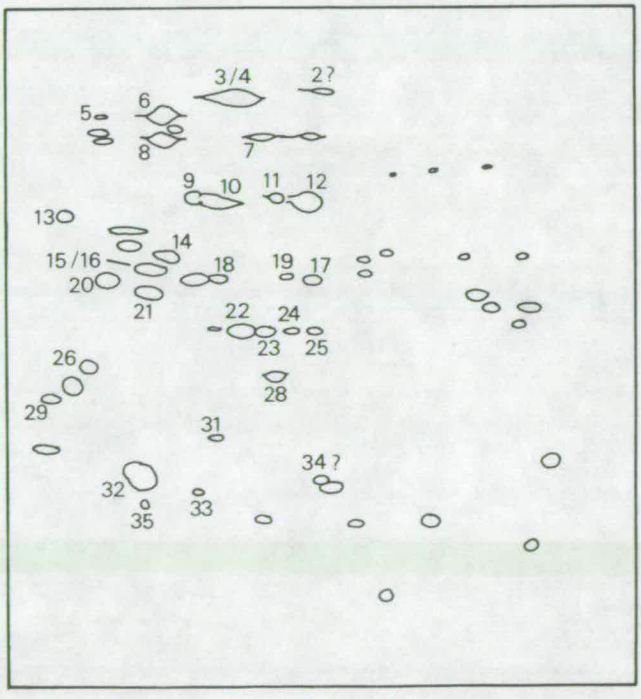
a) Labelling procedure

Exponentially growing cultures of 2-5% parasitaemia were incubated with labelled amino acids in appropriate medium for 16-24 hours at 37^{o}C in candle jars (section 2.1). At the end of the incubation period parasites were harvested by saponin lysis and solubilised in O'Farrell lysis buffer (section 2.3) before taking samples for scintillation counting. Two factors which were likely to affect the level of labelling parasite proteins in culture were i) the composition of the labelling medium, and ii) the length of the incubation period. Preliminary experiments were undertaken to assess the importance of these variables.

i) Composition of the labelling medium. Labelling experiments

Fig. 3.1 a) 2D gel of ^{35}S-methionine labelled proteins synthesised by saponin-released parasites (T9 clone 96). Proteins arrowed (↓) are more intensely labelled in extracts of labelled free parasites than in extracts of labelled cultures and those marked (▾) appear to be absent from the latter (cf. fig. 3.2).

b) Diagram of a) identifying those proteins (AT # 1-35) previously characterised on 2D gels by Tait (1981).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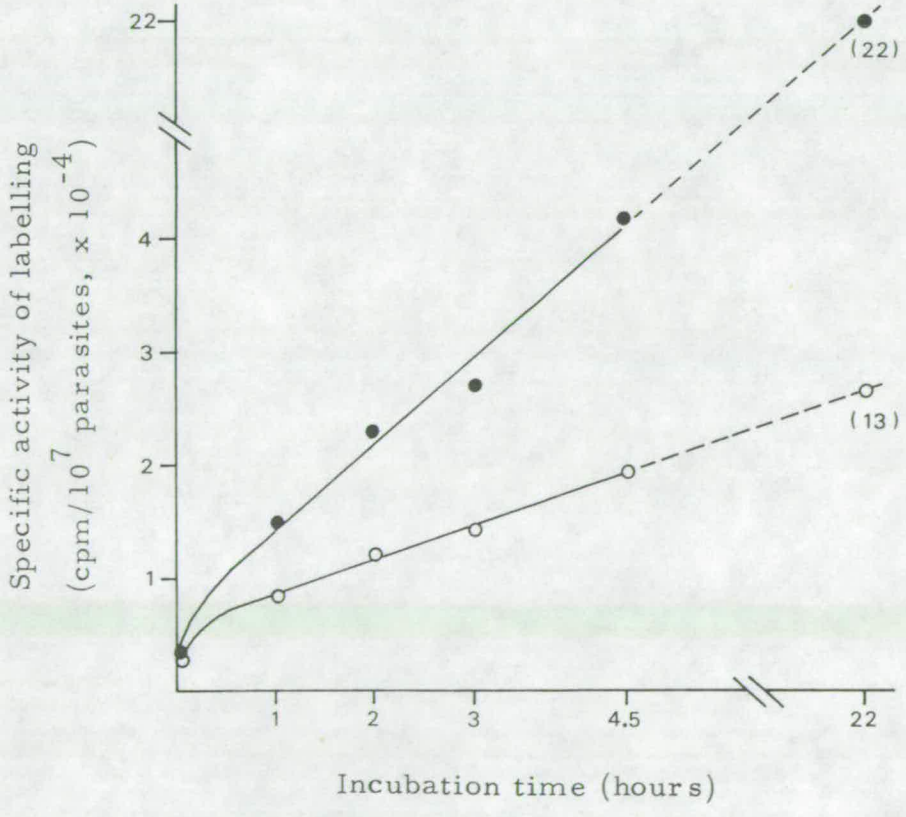
34?
32
33
35

were done in complete RPMI culture medium (section 2.1) or in medium in which the RPMI lacked the appropriate precursor amino acids. One experiment using a ^{14}C-protein hydrolysate is illustrated in Fig. 3.3 from which it can be seen that incorporation of label into protein was 2-3 times higher in RPMI lacking the amino acids in the labelled hydrolysate. A similar result was obtained using ^{3}H-methionine in that incorporation was 5-6 times higher in methionine-free RPMI than in complete medium. A second source of amino acids in the culture medium is the serum. Its presence was essential for good parasite growth and morphology and no benefit was gained by labelling in serum-free medium.

ii) Length of incubation period. It was found from the experiment shown in fig. 3.3 that the incorporation of labelled amino acids into protein appeared to continue at a constant rate over a 22 hour period in culture. Extending the labelling period to 40-48 hours (by 2 changes of labelled medium at 24 hour intervals) was attempted but, although higher specific activities of labelling were obtained, the total dpm recovered as protein was lower than for equivalent 20-24 hour incubations. This was due to the labelling medium not reliably supporting good parasite growth over periods of more than 24 hours. Thus the standard procedure adopted for labelling in culture was to use RPMI lacking the appropriate precursor amino acids and to incubate for periods of 20-24 hours.

Fig. 3.2 2D gel of ^{35}S-methionine labelled proteins synthesised by parasitised erythrocytes in culture (T9 clone 96) and recovered after saponin lysis. Proteins arrowed (↓) are more intensely labelled by this method than by labelling free parasite suspensions directly (cf. fig. 3.1).

Fig. 3.3 The effect of incubation in complete and precursor-free medium on the specific activity of protein labelled in culture. Cultures (5% parasitaemia, 7% haematocrit) were incubated with ^{14}C-protein hydrolysate (17 µCi/ml) in complete culture medium (o) or in precursor-free medium (•). 5 µl samples were removed at intervals to determine the TCA-precipitable cpm incorporated. Incorporation was approximately linear over the 22 hour labelling period and the final specific activities of labelling are given in brackets.


Specific activity of labelling
(cpm/10^7 parasites, x 10^-4)
22
4
3
2
1
(22)
(13)
1
2
3
4.5
22
Incubation time (hours)

b) Comparison of different labelled precursors

Two groups of labelled amino acids were tested. The first contained either ^{35}S-methionine or ^{14}C-labelled protein hydrolysate or a ^{14}C-mixture of five amino acids (leucine, lysine, phenylalanine, proline and tyrosine). The second group consisted of the equivalent ^{3}H-labelled amino acids. Details of the labelling conditions used and the levels of incorporation into protein observed are given in Table 3.2. Within both groups of precursors it can be seen that in terms of total dpm incorporated and specific activity of labelling, the five-amino acid mixture gave higher incorporation than methionine which in turn was better than the protein hydrolysate. With the exception of ^{35}S-methionine there was a strong correlation between the estimated total dpm incorporated (and the dpm/10^{7} parasites) and the effective specific activities of the precursors used. Despite the much higher specific activity of the ^{35}S-methionine the level of incorporation was about one tenth that of ^{3}H-methionine.

2D gels of extracts of cultures labelled with ^{35}S-methionine (figs. 3.2 and 3.4) and the ^{14}C - amino acid mixture (fig. 3.5) are illustrated. Gels of the equivalent ^{3}H-labelled proteins were identical to those shown of the ^{35}S- and ^{14}C- labelled cultures. Comparison of figs. 3.4 and 3.5 show that the 2D gel patterns produced by the two sets of precursors were almost identical. Amongst the 200 most intensely labelled proteins seen on the auto-radiographs all but one of the reproducible differences detected were small changes in the relative intensity of certain proteins (arrowed on

Table 3.2 Incorporation of labelled amino acids by cultures[i]

Labelled precursors	Specific[ii] activity, Ci/mmol	Nos. of exps.	Radioisotope concentration, μCi/ml	Parasite density, infected rbc/ml	Total protein labelled[iv], ave. dpm/ml	Specific activity of labelling, ave. dpm/10^7 parasites
^{35}S-methionine	920	10	13-33	2-4 x 10^7	8.0 x 10^5	3.0 x 10^5
^{14}C-protein hydrolysate	0.1-0.45	5	17-37	"	4.9 x 10^5	1.8 x 10^5
^{14}C-amino acid mix[iii]	0.25-0.52	4	13	"	19 x 10^5	7.1 x 10^5
^{3}H-methionine	87	8	17-67	2-4 x 10^7	6.7 x 10^6	3.4 x 10^6
^{3}H-protein hydrolysate	0.2-60	2	33-67	"	1.4 x 10^6	0.6 x 10^6
^{3}H-amino acid mix	70-170	2	50	"	19 x 10^6	7.8 x 10^6

(i) Cultures of parasitised erythrocytes were incubated with labelled precursors for 16-24 hours (Sections 2.2.2 and 3.2.2). (ii) For mixtures of amino acids, the range of specific activities of the individual amino acids is given. (iii) Contains leucine, lysine, phenylalanine, proline and tyrosine. (iv) See note (ii), Table 3.1. Incorporation was estimated after saponin lysis of the labelled culture and recovery of the free parasites.

figs. 3.4 and 3.5). Some of these proteins were more intensely labelled in the ^{35}S-methionine labelled extract and some more intensely in the ^{14}C-labelled sample. One major ^{14}C-labelled protein was apparently missing on gels of ^{35}S-methionine extracts.

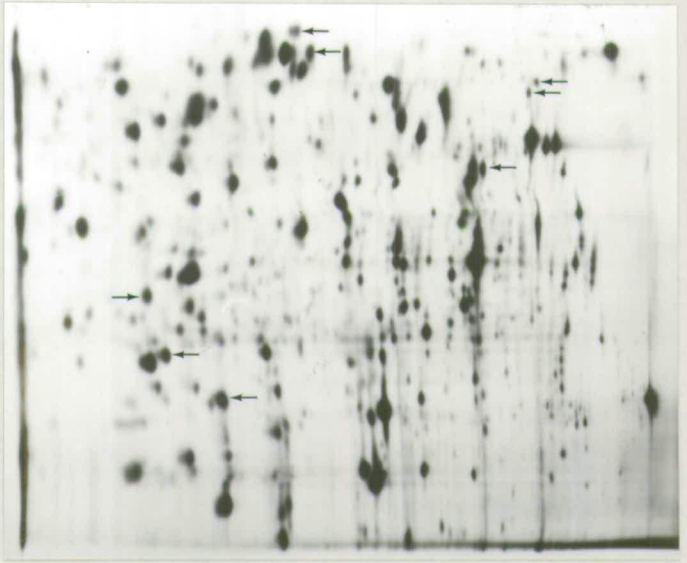
Many more differences were seen when parasites were labelled by different methods rather than by different precursors. The 2-dimensional gels illustrated in figs. 3.1 and 3.2 show samples of ^{35}S-methionine labelled free parasites and cultures respectively. It can be seen that although there is a strong overall similarity between the protein patterns there are some marked differences. Among the 200 most intensely labelled proteins visible on the gels there are major differences in the relative intensities of several proteins. Extracts of cultures have at least eight proteins which are more intensely labelled than in free parasites (arrowed on fig. 3.2). Conversely, at least 19 proteins were relatively more intense on autoradiographs of free parasite suspensions (arrowed on fig. 3.1). In addition, 6 proteins were seen in parasite suspensions which were either completely missing from extracts of labelled cultures or were present at too low a level to be detected (see fig. 3.1).

c) Loss of parasite protein during saponin lysis

The loss of parasite protein during the preparation of parasites from ^{3}[labelled cultures was investigated. Samples were taken at each stage of the saponin lysis procedure and TCA precipitated for scintillation counting (section 2.3.3). The results were used to

Fig. 3.4 2D gel of parasites (T9 clone 32) labelled in culture with ^{35}S-methionine. Those proteins indicated (↓) are more intensely labelled with ^{35}S-methionine than with ^{14}C-amino acids (cf. fig. 3.5).

Fig. 3.5 2D gel of parasites (T9 clone 32) labelled in culture with ^{14}C-amino acids (leucine, lysine, phenylalanine, proline and tyrosine). Proteins arrowed (↑) are more intensely labelled in ^{14}C-labelled extracts than in ^{35}S-methionine labelled extracts and the protein marked (*) was apparently not labelled with methionine (cf. fig. 3.4).



estimate the total amount of TCA-precipitable material present in the culture medium and, following saponin lysis, in the supernatant, the red cell ghost layer and the parasite pellet. These estimates are given in Table 3.3. Due to differences in counting efficiencies for different samples, these values are only very approximate.

TABLE 3.3 - LOSS OF LABELLED PROTEIN DURING SAPONIN LYSIS OF CULTURES

	% OF TOTAL TCA-PRECIPITABLE LABEL IN:			
Labelled Precursor	Culture Medium	Saponin Supernatant	Erythrocyte Ghosts	Parasite Pellet
^{3}H-methionine)) ^{14}C-amino acids)	10-20	40-60	5-10	20-40
^{35}S-methionine	50-75	10-15	1-2	5-10

3.3 Double-label Autoradiography

The method used was that of McConkey (1979). Comparison of two samples requires one to be labelled with ^{14}C or ^{35}S and the other with ^{3}H. The samples are mixed in the appropriate ratio and then run on a 2D gel (section 2.4). Following fixing, staining and fluorography (sections 2.5 and 2.6), two autoradiographic exposures are made which differentiate between the two isotopes as follows:

i) a fluorogram which detects both isotopes and therefore the proteins of both samples.

ii) a direct autoradiograph which detects only the higher energy

emitting isotope (^{14}C or ^{35}S) and therefore all the proteins of the ^{14}C (or ^{35}S) - labelled sample. Superimposing these two autoradiographs of the 2D gel allows those proteins present only in the ^{3}H-labelled sample to be identified since they appear on the fluorogram but not on the direct autoradiograph.

On the basis of the results presented in section 3.2 it was decided to label cultures of P. falciparum with the ^{3}H and ^{14}C-amino acid mixtures (leucine, lysine, phenylalanine, proline and tyrosine). This combination of labelling method and precursors was the most successful in terms of total incorporation of label into protein and the specific activity of labelling. It is necessary to adjust the amount of each labelled sample mixed together so that the proteins of each are detected at equal intensities by fluorography. As described by McConkey (1979) the greater the thickness of the SDS gel, the higher the ratio of dpm ^{3}H : dpm ^{14}C required for equal detection. Thus to keep down the total amount of ^{3}H-labelled protein required, gels of 0.75 mm thickness were used for all double-label gels. The correct ratio of cpm ^{3}H : cpm ^{14}C was determined empirically for this gel thickness and for the scintillation counting method used. Samples of a range of known cpm of ^{3}H and ^{14}C-labelled proteins were run separately on 1D SDS acrylamide gels. These were then processed and fluorograms developed as previously described. Examination of the autoradiographs determined that the cpm ^{3}H : cpm ^{14}C necessary for equal detection of labelled proteins under the conditions used was 6-7:1. This ratio was routinely used for

all double-label gels. The approximate exposure times were also determined empirically. Direct autoradiography required $2\frac{1}{2}$-3 times longer than fluorography for equivalent exposures. A gel run with 3×10^5 cpm of ^{14}C and 2×10^6 cpm of ^{3}H-labelled protein was exposed for 5-7 days to visualise about 200 protein spots by fluorography and for a further 14-20 days by direct autoradiography. Gels of 1.5×10^5 cpm ^{14}C and 1×10^6 cpm ^{3}H-labelled protein required 10-14 days and 28-40 days respectively.

In order to fully analyse two samples, gels of reciprocally labelled mixtures must be run. This is shown diagrammatically in fig. 3.6 for the comparison of the proteins of two strains of parasite - A and B. The two gels required are of ^{14}C-labelled A with ^{3}H-labelled B and of ^{3}H-A with ^{14}C-B. The fluorograph of the ^{14}C-A/^{3}H-B gel is shown in fig. 3.6 (a): both isotopes and therefore the proteins of both strains are detected. On the direct autoradiograph (b) of the same gel only the ^{14}C-labelled, strain A proteins are detected. Thus, those proteins present on the fluorograph but absent from the direct autoradiograph (arrowed in (a)) are found only in strain B. The remaining proteins are <u>either</u> present in both A and B <u>or</u> in A only. In order to distinguish between these two possibilities a similar analysis must be done for the reciprocally labelled ^{3}H-A/^{14}C-B gel. In this case proteins (arrowed in (c)) present on the fluorograph (c) but absent from the direct autoradiograph (d) must be unique to strain A. By combining the information from both sets of autoradiographs each protein can be unambiguously

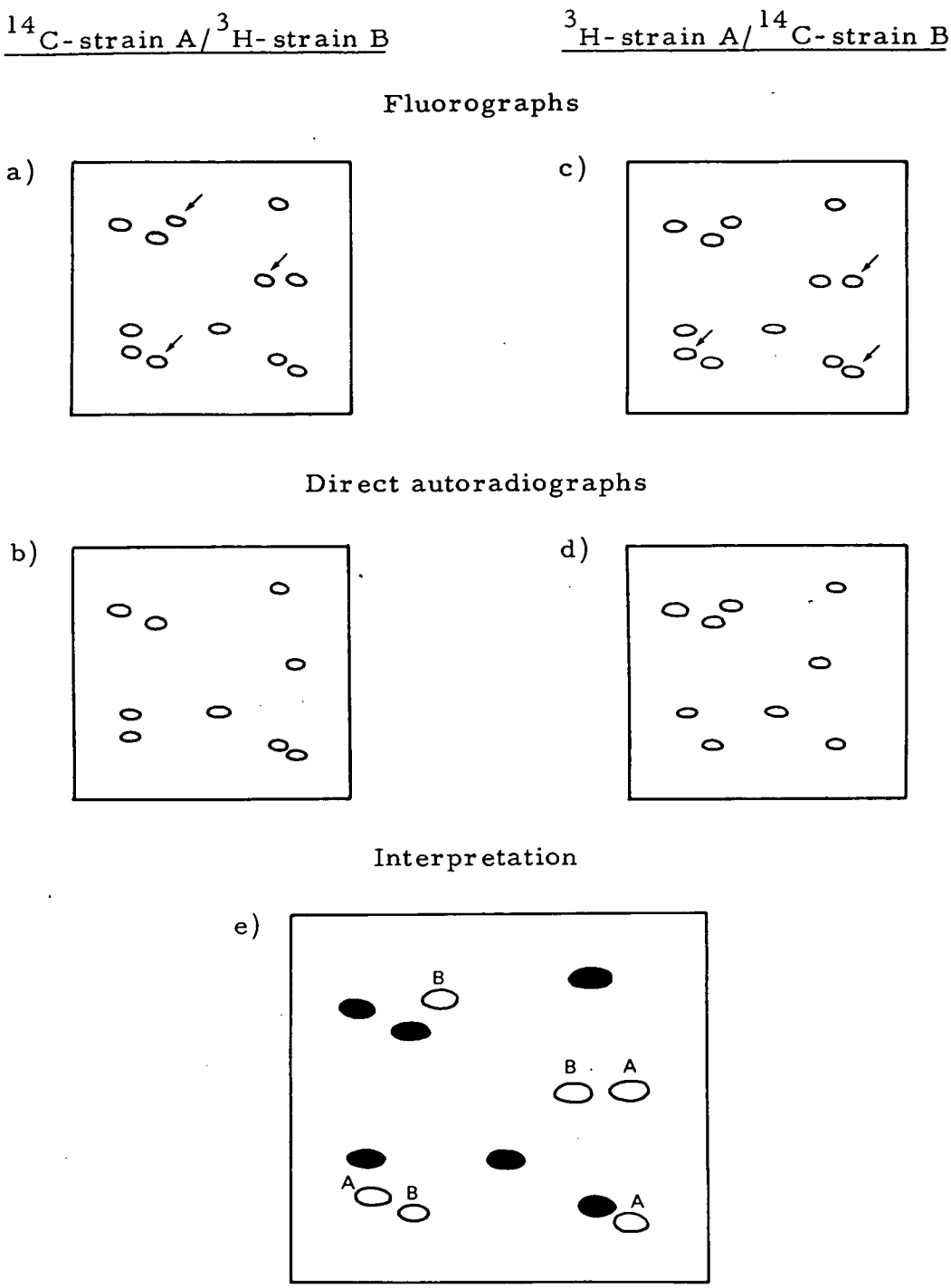
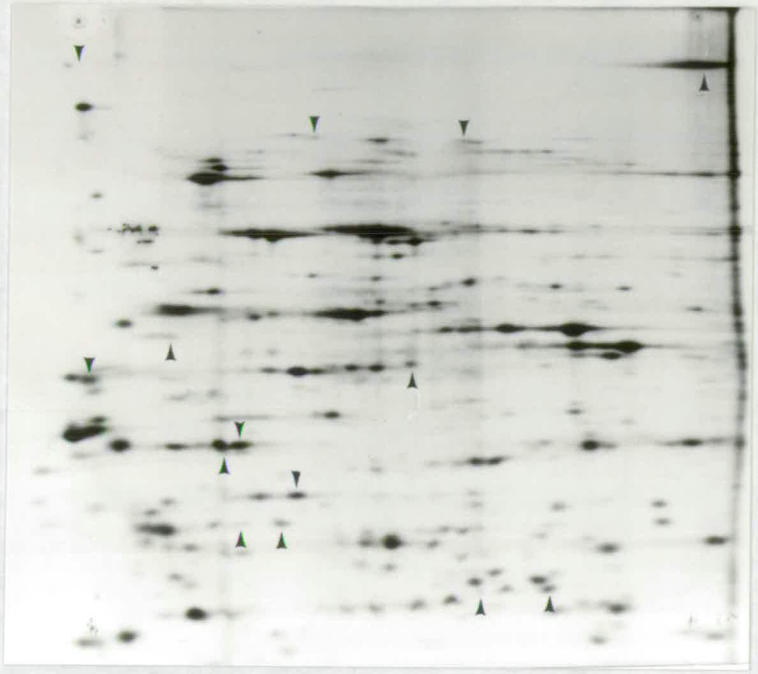


Fig. 3.6 Diagram illustrating the analysis of protein variation on 2D gels by double-label autoradiography. Spots arrowed in a) are specific to strain B and those arrowed in c) are specific to strain A. As shown in e), spots can be classed as present in one strain only (○, A or B) or common to both (●).

a)



b)

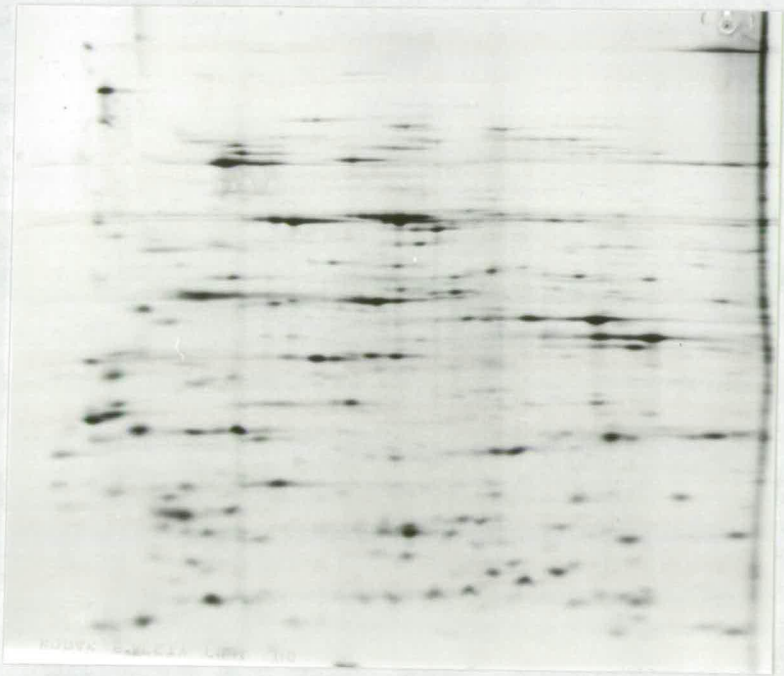
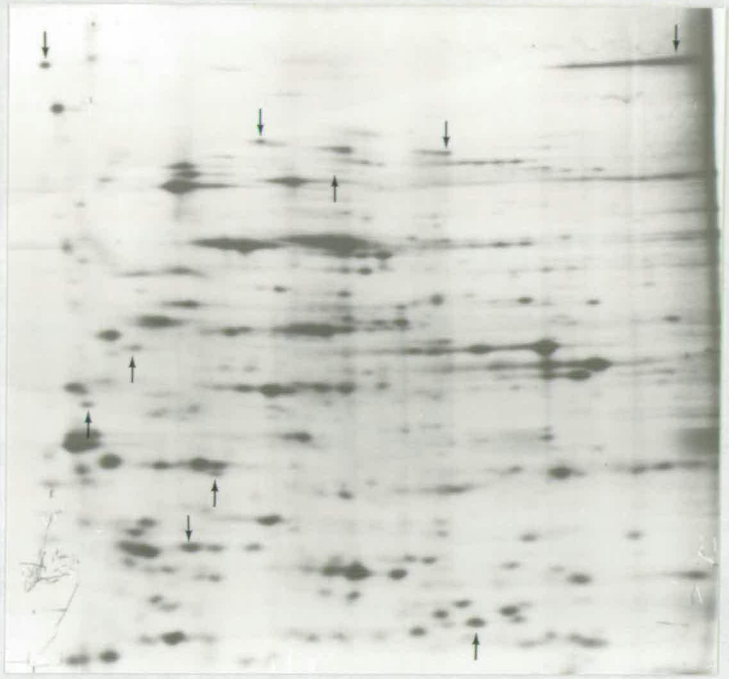


Fig. 3.7 a) is a fluorograph of a double-label gel of ^{3}H T17/^{14}C SK17. b) is a direct autoradiograph of the same gel. Proteins present only in a), i.e. which are specific to T17, are indicated (▾).

a)



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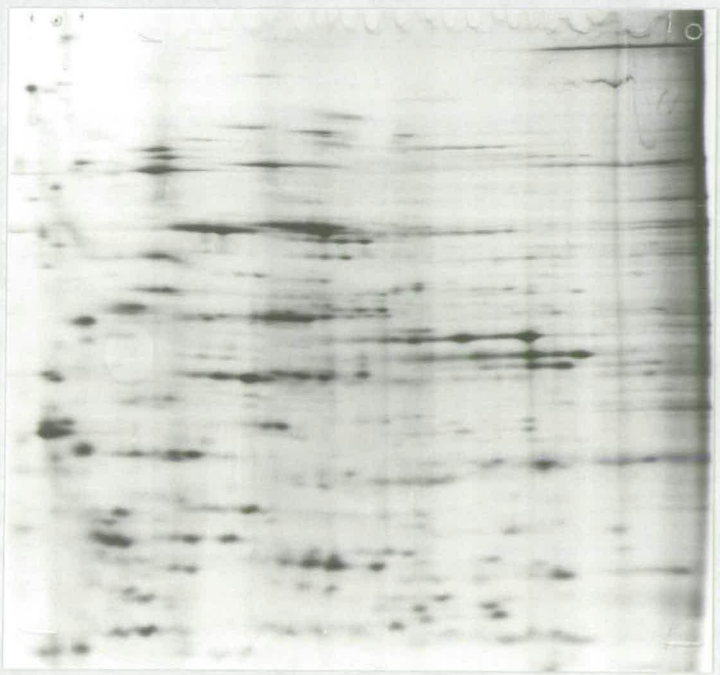


Fig. 3.8 a) is a fluorograph of a double-label gel of ^{3}H SK17/^{14}C T17. b) is a direct autoradiograph of the same gel. Proteins present only in a), i.e. those specific to SK17, are arrowed (↓).

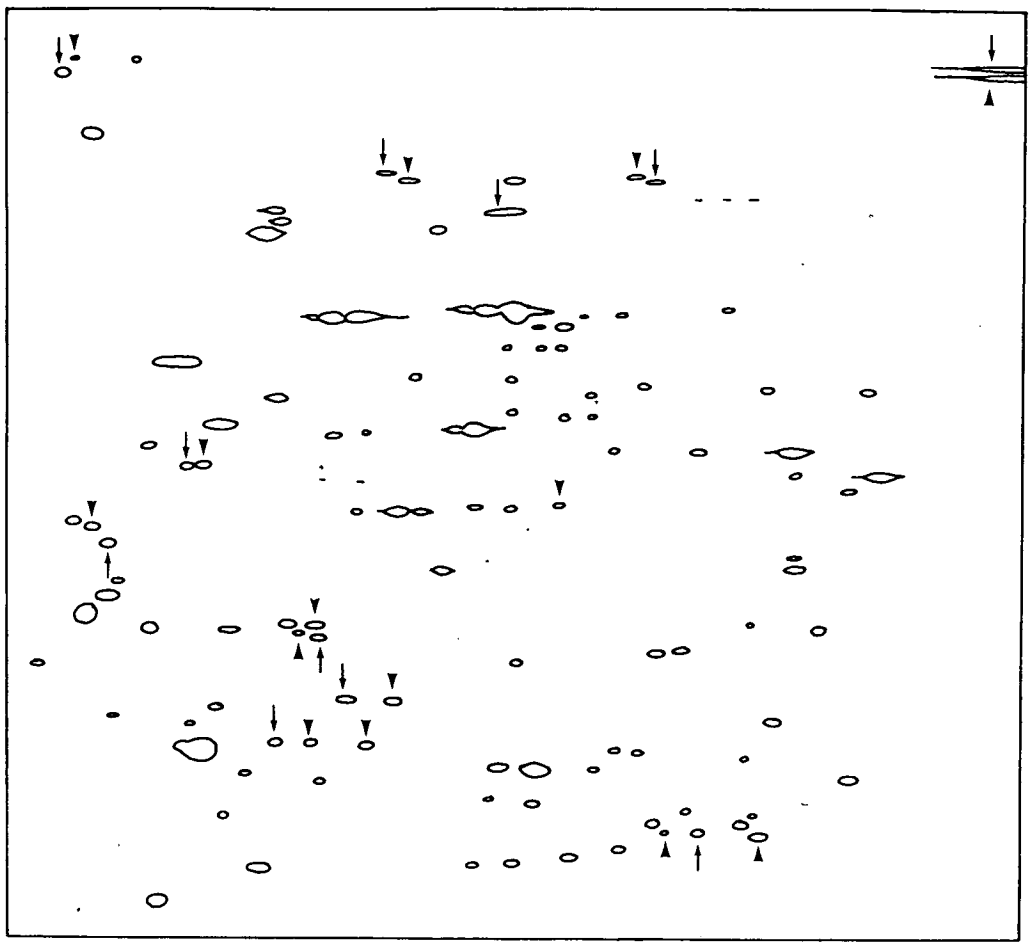


Fig. 3.9 Diagram showing differences detected between isolates SK17 and T17 by double-label autoradiography (see figs. 3.7 and 3.8). The major proteins seen on fluorograms of reciprocal double-label gels (figs. 3.7 a) and 3.8 a)) are illustrated. Those proteins arrowed (↓) are present only in SK17 and those marked (▾) are specific to T17. The remaining proteins are common to both isolates.

defined as being present in strain A only, in strain B only or in both A and B (fig. 3.6 (e)). Examples of actual double-label 2D gels of P. falciparum proteins and interpretative diagrams are shown in figs. 3.7 - 3.9.

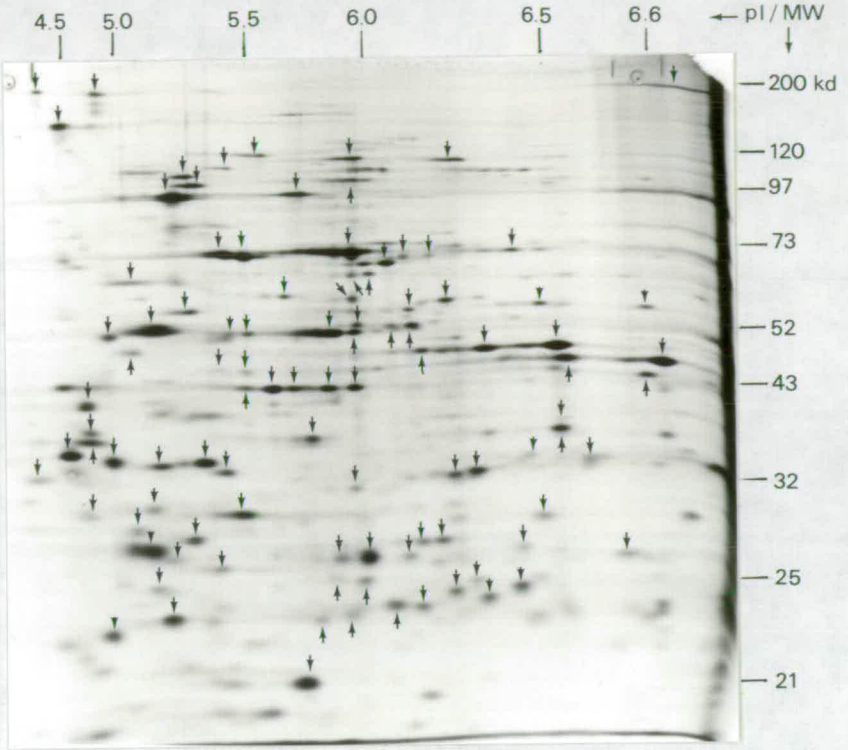
3.4 Characterisation of a set of major, reproducible P. falciparum proteins

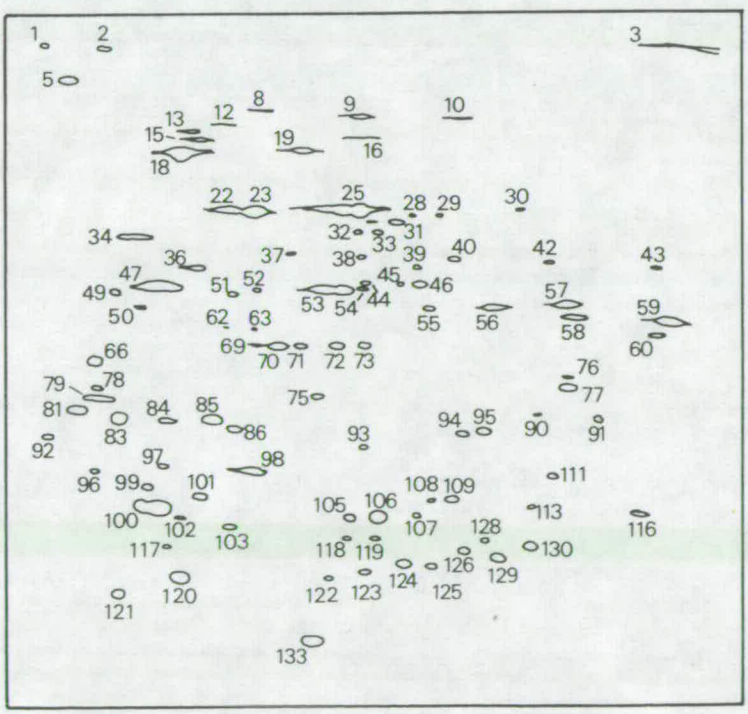
As a preliminary to analysing protein variation by 2DGE whether by single or double-label autoradiography it is necessary to examine the degree of reproducibility between gels. One P. falciparum isolate, SK17, was labelled in culture with the ^{14}C-labelled amino acid mixture on different occasions and each labelled extract was analysed on two or more 2D gels. Comparisons between gels were restricted to the most intensely labelled parasite proteins and a set of 100 proteins which appeared invariably on the SK17 gels are identified in fig. 3.10. The reproducibility between gels was generally high. It can be seen from fig. 3.10 that only a few strongly-labelled proteins were excluded from the set of reproducibly present parasite proteins. The 100 proteins were characterised in terms of their MW and pI by using direct pH measurements and creatine phosphokinase charge trains for calibration of the 1D gels and molecular weight marker proteins for the SDS gels (section 2.7; fig. 3.11). The MW and pI scales are shown on figs. 3.10 and 3.11 and the characteristics of all 100 proteins are given in Appendix 1.

The set of major, reproducible SK17 proteins (fig. 3.10) includes most of the 35 parasite proteins characterised in the earlier

Fig. 3.10 A representative gel of a ^{14}C-amino acid labelled culture of SK17 identifying the set of intensely labelled, reproducible proteins of this isolate. The MW and pI scales are shown.

Fig. 3.11 Diagram of the gel in fig. 3.10 showing the numbering system used throughout this thesis to identify the major, reproducible parasite proteins. The MW and pI of each protein is given in Appendix I.


4.5
5.0
5.5
6.0
6.5
6.6
pI / MW
200 kd
120
97
73
52
43
32
25
21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133

study of Tait (1981; fig. 3.1). However, in order to accommodate a larger array of proteins the numbering system used by Tait has not been adopted here. Instead, all the proteins characterised in this chapter and throughout the rest of this thesis (including some not classed as major, reproducible parasite proteins) have been collated and numbered roughly in order of decreasing MW and increasing pI. This allows specific proteins to be located relatively easily on 2D gels. In order to facilitate comparisons between the results of Tait and those presented in this thesis the identities of the proteins previously characterised by Tait (AT#1 - AT#35) are detailed in Appendix 1.

3.5 Discussion

Comparison of labelling methods

Two procedures for labelling parasite proteins were compared. The first was to incubate suspensions of parasites, which had previously been released from infected cultures, with labelled amino acids. The second was to label infected erythrocytes under normal culture conditions and then to harvest the parasites by saponin lysis.

The first method was thought to have some potential advantages over the second. The preparation of samples for gel electrophoresis is simpler and more rapid when dealing with labelled parasites rather than infected erythrocytes. Also, all the proteins synthesised by parasite suspensions should be present in the final preparation. Labelled cultures, on the other hand, may lose some

parasite proteins in the culture medium during the incubation period and further losses may occur during saponin lysis. Another possible advantage of incubating free parasites with labelled amino acids is that the parasites are in direct contact with the labelling medium. Incorporation of precursors into protein may therefore be more rapid than in parasitised erythrocytes where the uptake of labelled amino acids into, or dilution by, the intra-erythrocytic pool may limit the rate of incorporation (Sherman, 1977). However the use of saponin to release parasites prior to incubation with label may result in lysis or damage of the parasites which would have an adverse affect on incorporation. Also it can be argued that proteins labelled in cultures are likely to be more representative of the protein composition of the parasites in vivo. Post-synthetic modifications such as proteolytic processing and glycosylation may also be more likely to occur normally within the environment of the parasitised erythrocyte.

These methods of biosynthetically labelling parasite proteins were compared in terms of the efficiency of incorporation of labelled amino acids and of the 2-dimensional gel protein patterns produced. The levels of incorporation of ^{3}H-methionine achieved by these labelling methods are shown in Tables 3.1 and 3.2. These tables have been presented in such a way that the incorporation produced by equivalent μCi of precursor can be compared directly. However it should be noted that the actual number of parasites used for labelling in culture was about one tenth of that in parasite suspensions. The

results show that for similar ranges of μCi of ^{3}H-methionine, labelling in culture produced on average a slightly larger (x 2 - x 3) total of incorporated label (dpm) and a substantially higher (x 30 - x 40) specific activity of labelled protein (dpm/10^{7} parasites) in the final parasite preparation. This was despite losses of 60-80% of the labelled protein during incubation and saponin lysis of the cultures (Table 3.3). Therefore, the real level of incorporation may be 3-5 times higher in cultures than the estimates given. This high level of incorporation must be partly due to the much longer labelling period used. Longer incubations were feasible because a steady rate of incorporation was maintained over longer periods (> 22 hours) in culture than in parasite suspensions (~ 1 hour).

Although the incorporation of ^{3}H-methionine was substantially improved by labelling cultures rather than free parasites, that of ^{35}S-methionine was not. Comparison of the results in Tables 3.1 and 3.2 shows that cultures apparently produced lower amounts of labelled protein in the final extracts than did parasite suspensions. However there was an increase in the specific activity of labelled protein (x 3 - x 4). These differences between the results for ^{3}H- and ^{35}S-methionine may be associated with the higher percentage of TCA-precipitable dpm lost during the incubation and harvesting of cultures with ^{35}S-methionine (90-95%, Table 3.3) as compared with ^{3}H-methionine (60-80%). Comparison of the two labelling procedures indicated that labelling cultures is the better way of achieving high specific activities of labelled protein and, at least for some

precursors, of obtaining higher yields.

One of the disadvantages of labelling cultures was thought to be the possible loss of protein during the process. Table 3.3 suggests that a considerable percentage of incorporated label is lost either into the medium during incubation or into the supernatant during saponin lysis of the cultures. Loss of parasite protein into the culture medium is an expected consequence of labelling asynchronous cultures over long periods. Following schizogony only 4 or 5 of the 16-20 merozoites released successfully reinvade erythrocytes under culture conditions. Therefore, approximately 75% of the proteins present in mature schizonts will be lost into the medium during reinvasion. The concurrence of reinvasion and the appearance of labelled protein in the medium has been demonstrated for cultures of P. knowlesi (McColm, Shakespeare and Trigg, 1977) and P. falciparum (Rodriguez da Silva et al, 1983). As well as this general loss of protein there may be a selective loss. Any proteins which are specifically released into the culture medium, such as the S-antigens (Wilson and Bartholomew, 1975), or which are located in the erythrocyte membrane (Kilejian, 1979; Gruenberg and Sherman, 1983; Leech et al, 1984a) or cytoplasm may be lost during incubation or saponin lysis of the cultures. Such proteins would, however, be expected to be synthesised by, and present in, extracts of free parasite suspensions.

Two dimensional gels of proteins of labelled parasite suspensions and cultures of the same isolate are illustrated in figs. 3.1

and 3.2 respectively. As detailed in section 3.2, there are a number of differences between them. Some proteins were more intensely labelled or only present in extracts of free parasites, whereas a smaller number were more intensely labelled in preparations of labelled cultures. These changes could be a reflection of different rates of synthesis, degradation or processing of specific proteins in the two labelling regimes. However, the differences observed did not affect a random set of parasite proteins. The majority of proteins which were present or more strongly labelled in parasite suspensions were of high molecular weight (> 90 kd). Most, but not all, of those more intensely labelled in culture were of low molecular weight (20-35 kd). One possible explanation for the selective disappearance of high molecular weight proteins is that they may be predominantly located in the erythrocytic membrane or cytoplasm or in the culture medium and therefore lost during preparation of extracts of labelled cultures. (Evidence that this is true for at least some of these proteins is presented in Chapter 6). Another possibility is that saponin lysis may cause parasite membranes to become leaky and to lose proteins (Sherman, 1979). If certain parasite stages were affected to a greater degree than others then this could result in the loss of proteins specific to these stages.

Comparison of labelled precursors

The incorporation of precursors into protein in parasite suspensions will depend on the rate of synthesis and amino acid composition of the parasite protein and the concentration and specific

activity of the labelled amino acid. It can be seen from Table 3.1 that raising the specific activity of ^{3}H-methionine from 15 to 87 Ci/mmol gave approximately double the yield of labelled protein. Use of ^{35}S-labelled methionine which had a ten times higher specific activity did not however produce any more labelled protein than did equivalent μCi of the ^{3}H-labelled methionine. This may have been due to a correspondingly low concentration of methionine in the medium during labelling with the high specific activity precursor.

Incorporation of amino acids in infected cultures should also depend on the above factors. Additionally, incorporation may be affected by the rate of uptake of the amino acids into the erythrocyte and the extent to which the parasites can synthesise their own amino acids or obtain them from the proteolysis of haemoglobin (for review see Sherman, 1977). Three groups of amino acids were used for labelling P. falciparum cultures: ^{3}H- and ^{35}S-methionine, ^{3}H- and ^{14}C- protein hydrolysate and ^{3}H- and ^{14}C- mixture of leucine, lysine, phenylalanine, proline and tyrosine. The levels of incorporation for each are given in Table 3.2. There was a general trend of increasing incorporation with increasing specific activity of precursor under roughly equivalent labelling conditions. The five amino acid mixture gave the best yields and specific activities of labelled parasite protein.

The exception to the trend of high specific activity precursors producing high incorporation was ^{35}S-methionine. This gave lower yields of labelled protein than did ^{3}H-labelled methionine

which had one tenth the specific activity of the ^{35}S-precursor. Another difference between the two labels was that a greater percentage of TCA-precipitable material was found in the medium of ^{35}S-methionine labelled cultures than in that of either ^{3}H-methionine or ^{14}C-amino acids. There are two explanations of these observations. One is that in some way the ^{35}S-labelling conditions either increased the erythrocyte permeability or caused cell lysis and resulted in substantial losses of labelled protein into the medium. The other possibility is that the sulphydryl group has a different metabolic fate from that of the rest of the methionine molecule resulting in the sulphydryl group being diverted from parasite protein synthesis into other pathways. (For example, the ^{35}S may be transferred to cysteine and then into the tripeptide glutathione which could account for some of the TCA-precipitable counts in the culture medium).

When 2-dimensional gels of proteins from cultures labelled with methionine or the five-amino acid mixture were compared (figs. 3.4 and 3.5) only a few minor differences in intensity of labelling were detected. Those proteins labelled more intensely on the ^{35}S-methionine gels presumably represent methionine-rich proteins. Those labelled more intensely by the ^{14}C- amino acids may be proteins poor in methionine. One protein which was apparently missing on the ^{35}S- gel probably lacked methionine in its primary structure.

When these gels are compared with those of proteins labelled

by the alternative methods (figs. 3.1 and 3.2) it can be seen that more marked differences in the two-dimensional patterns were produced by changing the labelling method than was effected by varying the labelled precursors.

Double-label autoradiography

As mentioned in the section 3.1 the main drawback of a double-label system for analysing two-dimensional gels is that the autoradiographic exposure times can be lengthy. Section 3.3 showed that in order to keep exposure times for fluorography to within 1-2 weeks and for direct autoradiography to within 3-6 weeks, it was necessary to have 1-2 x 10^6 cpm (5-10 x 10^6 dpm) of ^{3}H-labelled protein and 1.5 - 3 x 10^5 cpm (2 - 4 x 10^5 dpm) of ^{35}S- or ^{14}C-labelled protein. It was initially hoped to produce material for double-label autoradiography by incubating free parasites with ^{3}H- and ^{35}S-methionine. However, it was not possible to incorporate enough ^{3}H-methionine by this method to satisfy the above requirements. Scaling up the incubation mixture could have produced the cpm of labelled protein needed but at the cost of doubling or trebling the amount of isotope used and the concentration of protein applied per gel.

After investigating alternative labelling methods and precursors (Tables 3.1 and 3.2) it was found that labelling in culture with equivalent μCi of amino acids gave better yields of incorporated label with lower densities of parasites. Best results were obtained with

the ^{3}H- and ^{14}C- mixture of five amino acids. Samples for double-label gels could be successfully made using only about one half the μCi of label and about one tenth the number of parasites which would have been needed for labelling free parasite suspensions to the required level. Therefore, the combination of the ^{3}H- and ^{14}C-amino acids and labelling in culture was routinely used to provide samples for double-label autoradiography.

The 2D gel analysis of parasite proteins

The other aspect of the work described in this Chapter was to assess the reproducibility of the protein profiles produced by 2DGE. If 2D gels are to be used to investigate protein variation in different strains of parasite then it is clearly essential to ensure that the proteins compared are reproducibly present and do not vary electrophoretically in different samples of the same parasite isolate. By analysing several labelled preparations of the Thai isolate, SK17, it was found that the reproducibility between gels was generally high and a set of 100 intensely labelled, reproducible proteins were selected and characterised in terms of their MW and pI. This set of standard, or reference, proteins serves two functions. Firstly, by providing a set of internal MW and pI markers, it allows other proteins and new electrophoretic variants seen on 2D gels to be easily characterised. Secondly, this array of parasite proteins provides the necessary framework for the more extensive 2D gel studies of P. falciparum described in the rest of this thesis.

THE ANALYSIS OF PROTEIN VARIATION IN ISOLATES OF P. FALCIPARUM

4.1 Introduction

The analysis of proteins which vary between isolates is one of the main objectives of this thesis. Once such variation has been characterised it has several practical applications, the most obvious being the identification or "fingerprinting" of different isolates of P. falciparum. Variant proteins may also be of value for identifying isolates which are mixtures of parasite genotypes and for confirming successful cloning of cultures. Protein variants may be suitable genetic markers for investigating the population structure of the parasite and for future use in genetic analysis. Furthermore, the identification of variation in proteins involved in immunity or drug resistance may have important implications for the medical aspects of malaria research.

Two-dimensional gel electrophoresis (2DGE) was first applied to the analysis of protein variation among isolates of P. falciparum by Tait (1981). Although only 35 major parasite proteins were examined, 14 of these were found to vary electrophoretically in some of the seven isolates analysed. The results demonstrated that sufficient variation occurred to allow each isolate to be distinguished from the others. Also, comparison of the protein patterns of isolates from the Gambia, W. Africa and from S.E. Asia suggested that isolates from these two regions could be differentiated from each other by having specific variants of certain proteins. There was

also some indication that regional variation may occur within S.E. Asia. However, only a few isolates were screened for a relatively small number of proteins. Thus, one aim of the work reported here was to extend the two-dimensional gel analysis by screening more isolates and using a larger set of proteins. The approach used was to combine gel electrophoresis with double-label autoradiography as detailed in Chapter 3. Following the identification and characterisation of the variation detected, the aims were to use the information

i) to type a number of parasite isolates,

ii) to identify those isolates which may be of mixed parasite genotypes, and

iii) to look for further evidence of regional variation within P. falciparum.

4.2 Two-dimensional gel analysis

The isolates under study originated from different geographical areas and comprised 14 from three regions of Thailand, 6 from Papua New Guinea and 1 from the Gambia, West Africa. Details of these isolates are given in section 2.1.

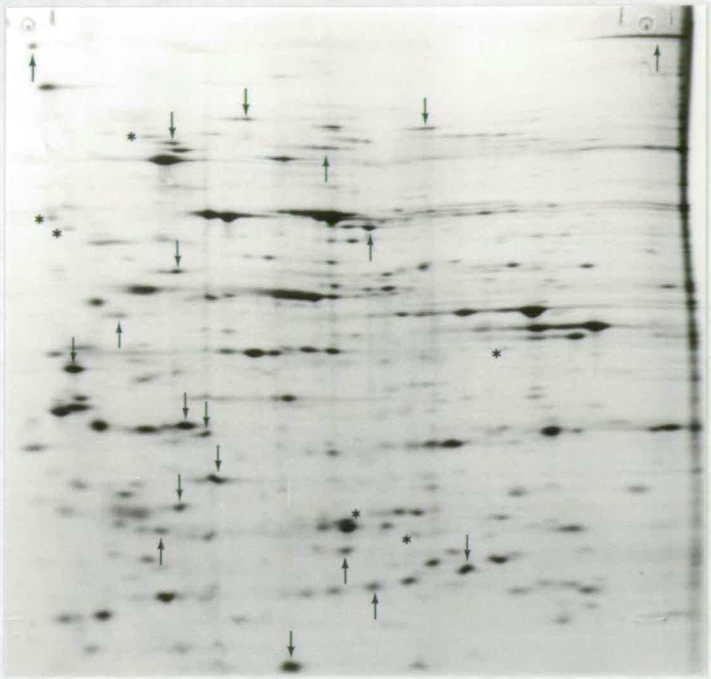
In order to increase the accuracy and efficiency of detecting differences between isolates on two-dimensional gels, double-label autoradiography was used. One of the Thai isolates, SK17, was arbitrarily chosen as a reference isolate and all the other isolates were analysed by making pairwise comparisons with SK17. Each

isolate to be screened was labelled separately with the ^{3}H- and ^{14}C-amino acids and reciprocal double-label gels were set up with SK17 as the ^{3}H-labelled sample on one gel and as the ^{14}C- sample on the other. The final autoradiographs were analysed and interpreted as explained in section 3.3 and as illustrated in figs. 3.6 - 3.9. The proteins of other isolates were thus compared directly with those of the standard isolate, SK17, and any differences were catalogued. Comparisons were based on the set of major, reproducible SK17 proteins which were identified previously (figs. 3.10 - 11). All the cultures analysed were asynchronous. Blood smears of most of these cultures were made before and after labelling to monitor both the growth and the stage-distribution of the parasites. Examination of the smears showed similar proportions of the different parasite stages in most cultures. The results of this screening programme are presented below.

4.3 Protein Variation

The 100 major proteins seen reproducibly on gels of SK17 are shown in figs. 3.10 and 3.11. 81 of these proteins were also seen in identical positions on two-dimensional gels of all the other isolates examined. These invariant proteins are identified in fig. 4.1. The remaining 19 proteins, arrowed in fig. 4.1, were not invariably present. 18 of these proteins were absent from some isolates but were always replaced by another in the same region of the gel and so are

a)



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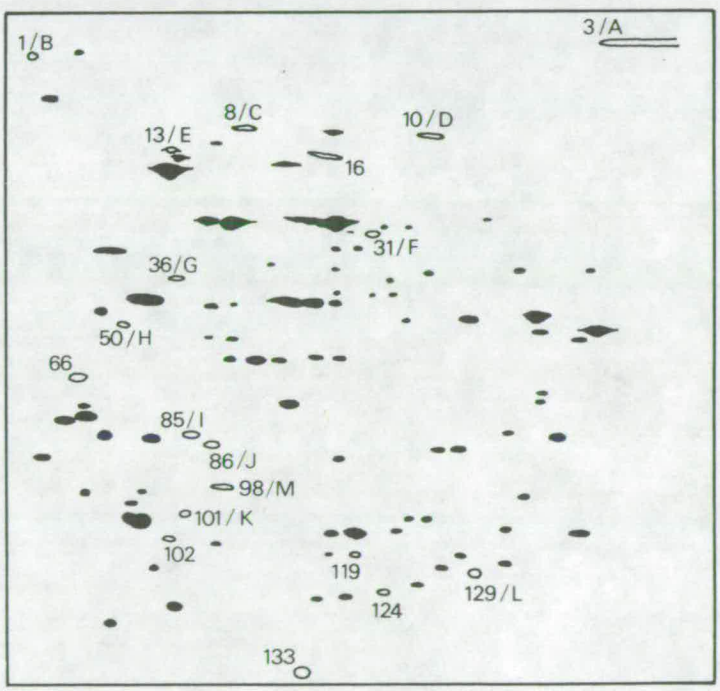


Fig. 4.1 a) 2D gel of ^{14}C-labelled SK17. Members of the set of major, reproducible proteins (defined in figs. 3.10-11) which vary between isolates are arrowed (↓). Other minor proteins observed to vary between isolates are also indicated (* , see Appendix 2). b) Diagram of a) identifying the major, reproducible SK17 proteins. ● , invariant proteins. ○ , strain-variant proteins. Proteins A-M used for strain typing are marked.

almost certainly electrophoretic variants of the proteins seen in SK17. Within this group of variable proteins, seven had variants which differed in isoelectric point only (#31, 85, 86, 98, 119, 129 and 133) and eleven had variants which differed in isoelectric point and molecular weight (#1, 3, 8, 10, 13, 36, 50, 66, 101, 102 and 124). Both types of variation can be seen in fig. 4.2.

Two further types of variation were observed. Firstly, the presence or absence of certain proteins was noted. One of the SK17 proteins (#16) was completely missing from some isolates. Other instances were noted where proteins which had not been seen in SK17 appeared reproducibly on gels of other isolates. Secondly, a further difference between isolates was a change in the relative intensity of some proteins on the autoradiographs. For example, proteins #107 and #123 varied noticeably in intensity on gels of certain isolates.

However, for purposes of characterising isolates only those proteins which apparently varied electrophoretically were considered further. Variable proteins were selected as suitable for isolate typing by the following criteria:

i) Putative variants should, like the equivalent SK17 proteins, be reproducible in presence and position. ii) New variants should be of similar relative intensity and iii) in close proximity to the corresponding protein spot of SK17. These conditions should increase the probability that the electrophoretic shifts attributed to a protein actually reflect genetic variation in that protein rather than the

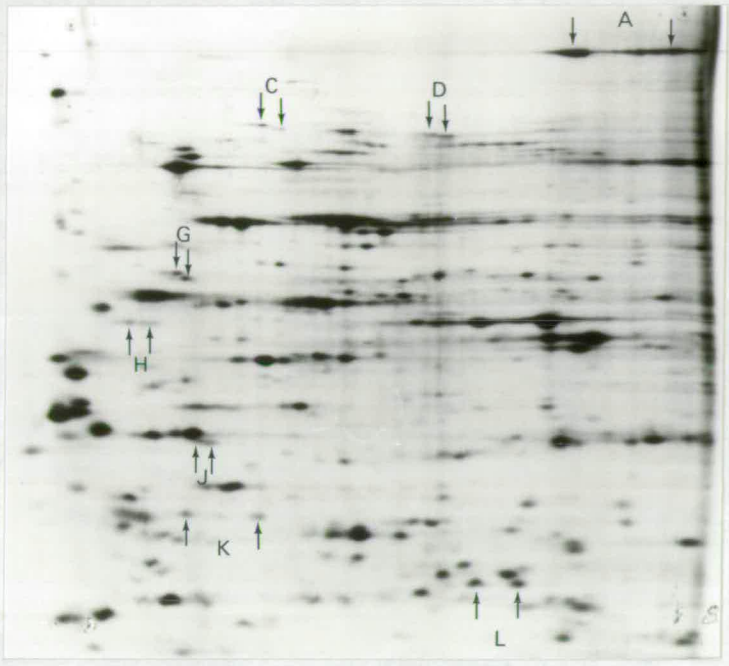


Fig. 4.2 Fluorograph of a double-label gel of ^{3}H K29/ ^{14}C SK17. Two variants of proteins A, H, J, K and L each differing in pI can be seen. Variants of C, D and G differ in both pI and MW.

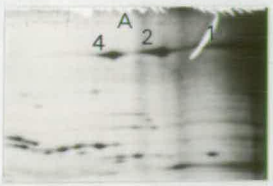
chance appearance of an unrelated protein or some post-synthetic modification.

Five variable proteins did not comply with these criteria. Four (#66, 102, 119, 124) did not satisfy condition (i) and one (# 1) satisfied neither (ii) nor (iii). A further protein (# 133) was not used as a character for typing because it was of low molecular weight (approximately 20 kd) and as a result did not appear on all gels.

Twelve proteins did satisfy the above criteria and were used for typing isolates. In addition protein # 1, despite not satisfying these conditions, was included because it had previously been shown to be extremely variable (Tait, 1981) and therefore of considerable value for strain typing. Thus a total of thirteen proteins were used for isolate characterisation (# 1, 3, 8, 10, 13, 31, 36, 50, 85, 86, 98, 101, and 129). To avoid confusion with the nomenclature used by Tait, 1981, these variable protein positions have been lettered (A – M, fig. 4.1, Table 4.2) and the electrophoretic variants of each protein numbered in order of discovery.

The number and relative positions of the different variants of each protein are illustrated in fig. 4.3 and their MWs and pIs are detailed in Table 4.1. It can be seen that there were three main patterns of electrophoretic variation, as outlined below:

a) Proteins F, I, J, M and L (i.e. # 31, 85, 86, 98 and 129 respectively) had variants which differed in isolectric point only. Only two variants were identified for proteins I, J, M and L. Protein F had three variants and, as shown in fig. 4.3, the more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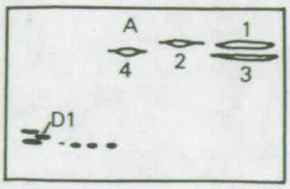
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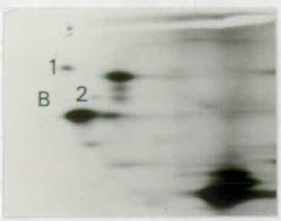
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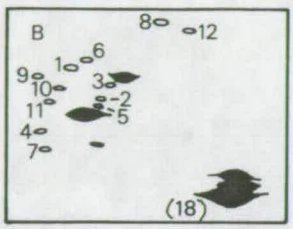
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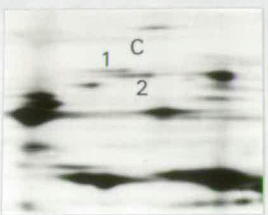
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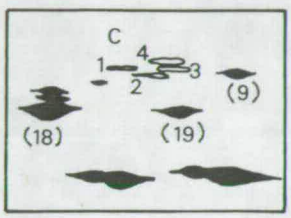
C
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3
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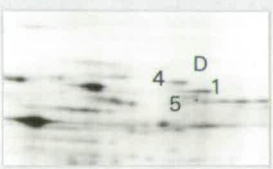
(9)
(18)
(19)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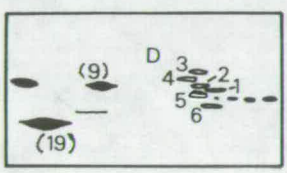
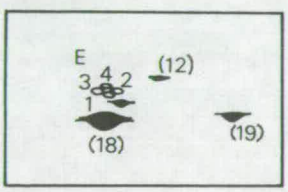
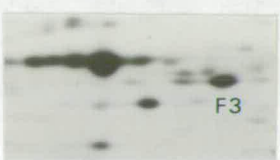
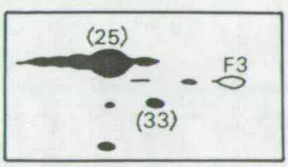
1
5

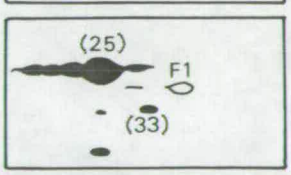

(9)
D
3
4
2
1
5
6
(19)

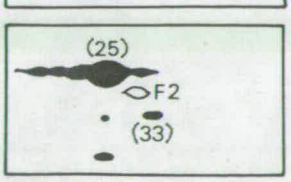

E
3
2


E
(12)
3
4
2
1
(18)
(19)


F3


(25)
F3
(33)


(25)
F1
(33)


(25)
F2
(33)

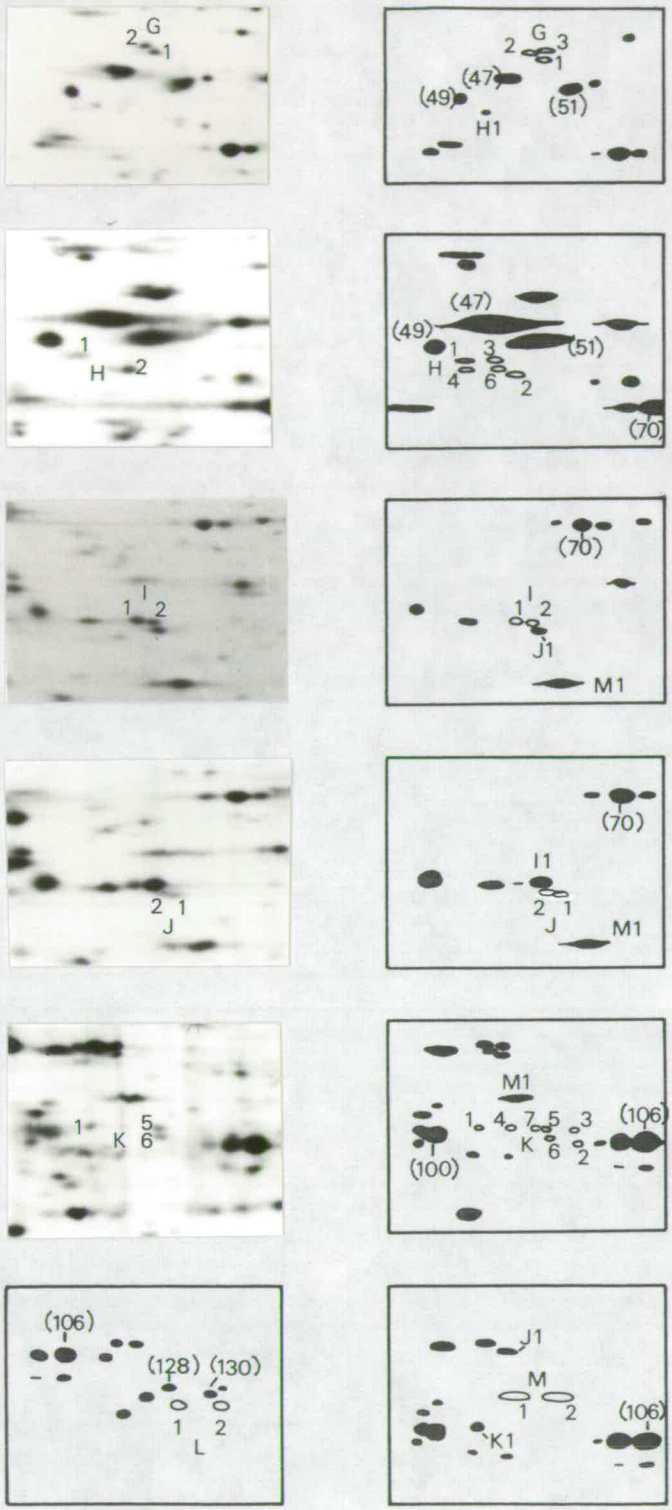


Fig. 4.3 Electrophoretic variants of proteins A-M. Sections of 2D gels illustrate two or more variants of each protein. The diagrams detail the relative positions of all variants (open spots) of each protein. Neighbouring proteins are also shown (filled spots) and the identity of invariant proteins is given in brackets. Gels showing variants L1, L2, M1 and M2 are not given as these are clearly illustrated in figures 4.5 - 4.10.

basic variants had additional faintly labelled spots at the positions of the more acidic variants. The electrophoretic shifts observed were measured by comparing them with the spacing of the creatine phosphokinase charge isomers in the same region of the gel (section 2.7) or by estimating the pI differences between the variants. For all these proteins, with the probable exception of M, the results suggest that the shifts observed were consistent with the expected effect of single charge differences between the variants (see Table 4.1).

b) The second type of variation was seen in proteins A, C, D, E, G, H and K (#3, 8, 10, 13, 36, 50 and 101). All had some variants which differed from each other in pI or MW only but also had two or more variants which differed in both pI and MW simultaneously. Within this group of proteins some were more variable than others. For example, six or seven variants were identified for each of D, H and K but only three or four for each of A, C, E and G. Different patterns of electrophoretic variation were also seen (fig. 4.3). Protein K, for instance, had more variants differing in pI than in MW whereas all the variants of D differed from each other in MW.

In absolute terms, the molecular weight differences between successively larger variants of each protein showed a tendency to increase with the size of that protein. Thus, variants of A, the largest of these proteins (~200 kd) differed by 8-10 kd; variants of C and D (both ~120 kd) by 2-7 kd; variants of E (~110 kd),

Table 4.1 Molecular weights and isoelectric points of variants of proteins A - M

Protein		Electrophoretic variant: 1	2	3	4	5	6	7
A	MW(kd)	196	196	188	188			
	pI	>6.6	~6.6	>6.6	6.47			
B*		200	169	174	135	157	206	125
		<4.5	4.76	4.82	<4.5	4.60	4.50	<4.5
C		122.6	120.5	121.9	124.5			
		5.60	5.68	5.74	5.72			
D		116.9	117.2	124.7	122.5	113.8	106.4	
		6.31	6.26	6.26	6.24	6.28	6.31	
E		107.2	107.9	108.9	110.2			
		5.33	5.36	5.30	5.34			
F		69.7	69.7	69.7				
		6.05	5.96	6.15				
G		56.9	58.3	57.7				
		5.35	5.31	5.35				
H		48.4	47.0	48.6	47.6		47.5	
		5.08	5.24	5.22	5.09		5.21	
I		34.1	34.0					
		5.38	5.45					
J		33.2	33.5					
		5.46	5.39					
K		27.9	26.7	27.8	27.9	27.9	27.4	28.0
		5.36	5.76	5.74	5.46	5.61	5.62	5.58
L		24.5	24.4					
		6.41	6.46					
M		29.8	29.8					
		5.49	5.64					

	8	9	10	11	12
*B	>250	176	171	156	>250
	5.07	<4.5	<4.5	<4.5	5.21

Note: The pI's, although only accurate to ±0.1 units, have been estimated to 0.01 in order to give a better measure of the charge differences between variants of each protein.

G (~57 kd) and H (~48 kd) by approximately 1 kd; and those of the smallest protein, K (~28 kd), by less than 0.5 kd.

In the case of proteins A, C, D, E and G the shifts in pI were roughly equivalent to those of unit charge changes between variants. Those of H and K, on the other hand, appeared to be of greater magnitude.

c) The third type of protein variation was that seen among the high MW, acidic proteins grouped together as B although it is not clear whether or not these are variant forms of the same parasite protein. As many as twelve putative variants were identified and these differed widely in both pI (< 4.5 - 5.2) and MW (125 -> 250 kd). Unlike the other proteins chosen for strain typing, the variants of B differed markedly in relative intensity of labelling. In five isolates, e.g. K36, no variant of B could be identified on the 2D gels. However, it is possible, in view of the extreme MW and pI range of protein B, that variants occurred in these isolates which were too acidic to focus in the first dimension pH gradient or too large to enter the 10% acrylamide gel in the second dimension.

Further information is provided in Appendix 2 about variation in proteins which were not used for isolate characterisation.

4.4 Isolate Typing

The isolates were characterised by analysing the thirteen variable proteins (A – M) described above and determining which variant of each protein they possessed. The results are set out in Table 4.2 and two-dimensional gels of several isolates, showing the

Table 4.2 Protein variants of P. falciparum isolates

Origin of isolate	Isolate	Variant proteins A	B	C	D	E	F	G	H	I	J	K	L	M
Songhkla, Thailand	SK15	1	2	2	1	2	1	2	2	1	2	2	2	1
	SK16	1	2	2	1	2	1	2	3	1	2	7	2	1
	SK17	1	1	1	1	1	1	1	1	1	1	1	1	1
	SK18	2	3	2	2	2	2	2	1	1	2	1	1	1
	SK19	2	3	2	2	2	2	2	1,4	1	2	1	1	1
Kanchanaburi, Thailand	K1	4	-	2	4	2	1	1	3	1	2	5	2	1
	K28	2	4	2	2	4	3	1	1	1	2	4	2	1
	K29	2	-	2	2	2	1	2	3	1	2	5	1,2	1
	K36	2	-	2	3	2	1	2	1,3	1	2	1	2	1
Mae Sod, Thailand	T9	2,4	7,8	2,3	2,5	2,3	2,3	2	3,6	1,2	1	3,6	1	1
	T17	3	6	2	2	2	1	1	3	1,2	2	4,7	2	2
	T19	3	-	1	6	2	3	1	6	1	1	6	2	1
	T20	2	-	1	4	3	1	1	1	1	1,2	4	2	1
	T22	3,4	5	2	3	2	3	1,2	2	1	1	2	1	1
Papua New Guinea	FCQ2, MURRI, MAD 13,20,22,26	2	1,12	2	4	2	1	2	3	1	2	7	1	1
Gambia, W. Africa	G1	2	9,10,11	4	4	3	1	1,3	3	1	1	3	1	1,2

Part of these results have previously been published in Walliker (1983a,b). A number of differences between the above table and the earlier version, including changes in nomenclature, are detailed in Appendix 3.

variant proteins, are illustrated in figs. 4.5 - 4.10.

It can be seen from Table 4.2 that the majority of the isolates screened had a unique combination of protein variants and could therefore be unambiguously distinguished from each other. The exceptions to this were the six Papua New Guinea isolates which all appeared identical. The degree of similarity between the other isolates varied greatly. This is shown quantitatively in fig. 4.4 which shows the number of proteins differing in all possible pairwise comparisons of the isolates typed. It can be seen that some isolates were very similar to each other. For example, SK18 and SK19 were identical in regard to twelve of the thirteen proteins and differed only in SK19 having an extra variant of protein H. SK15 and SK16 differed in only two proteins (H and K). At the other extreme, SK15 and G1 had only three variant proteins in common (F1, I1 and M1) and differed at the remaining ten.

Some isolates had more than one variant of certain proteins. SK19 had variants H1 and H4; K29 had L1 and L2; and isolates K36, T17, T20, T22, the Papua New Guinea isolates and G1 all had two variants of one or two variable proteins. Most striking in this respect was T9 which had two variants of each of nine proteins. If it is assumed that these variants are genetically determined then, since the bloodstream parasites are haploid, these results can be interpreted as indicating that the above isolates are mixtures of parasite genotypes. This interpretation has been shown to be true for isolate T9. Cloned lines derived from T9 had only one variant

a)

	SK15	SK16	SK17	SK18	SK19	K1	K28	K29	K36	T9	T17	T19	T20	T22	PNG
SK16	2														
17	8	8													
18	7	7	8												
19	7	7	8.5	0.5											
K1	6	5	9	8	8										
28	8	8	9	6	6.5	7									
29	5.5	4.5	9.5	4.5	4.5	3.5	6.5								
36	5	4.5	8.5	4.5	4.5	4.5	6.5	3							
T9	10.5	9	9.5	7	7	9.5	9.5	7.5	8.5						
17	7.5	6	10.5	8.5	8.5	5.5	7	6	7	10.5					
19	9	9	8	10	10	7	8	8.5	8	8.5	8.5				
20	7.5	8.5	6.5	8.5	9	5.5	5.5	7	6	10	8.5	6.5			
22	6.5	8.5	8.5	8.5	8.5	8	8.5	8	8.5	7	9.5	7	10		
PNG(i)	6	4	8.5	5	5	5	8	3.5	4.5	7.5	7	10	7.5	7.5	
G1	10.5	9.5	8	9.5	9.5	8	10	8	7	8	9.5	10	6.5	9	7.5

b)

	SK	K	T	PNG
SK	6.3			
K	6.5	5.2		
T	8.4	7.4	8.6	
PNG	5.7	5.2	7.9	-
G1	9.4	8.8	8.6	7.5

Fig. 4.4 a) details the results of pairwise comparisons of all possible combinations of the isolates typed in Table 4.2. Analysis was restricted to proteins A-M and the number of proteins which had different variants in each pair of isolates is shown. (Where an isolate had two variants of a given protein, one identical to and one different from that of a second isolate, then this was scored as a difference of 0.5).

In b), intra- and inter-group comparisons have been averaged to assess the extent of the intra- and inter-regional variation among these isolates.

(i) PNG = the identical FCQ2, Murri, MAD 13, 20, 22, and 26 isolates.

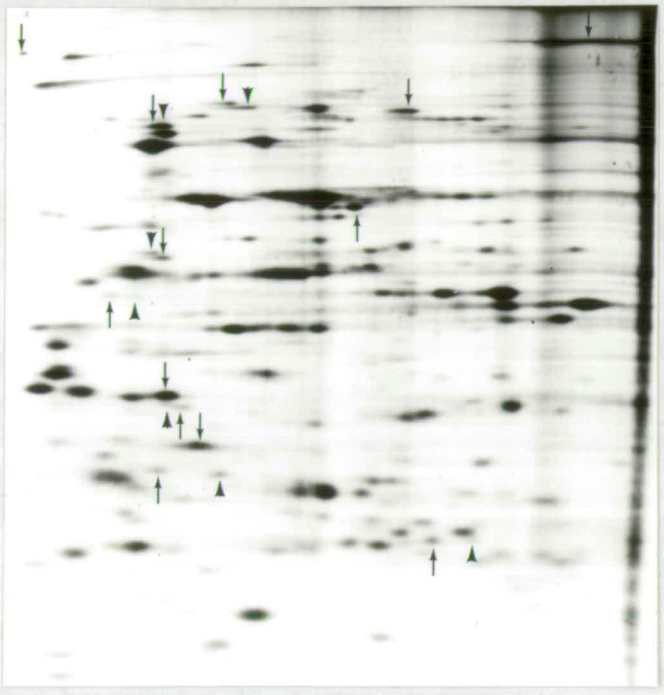
Figs. 4.5-4.10 Fluorographs (a) and direct autoradiographs (b) of double-label gels of ^{3}H-SK17 and ^{14}C-SK16 (fig. 4.5), ^{14}C-SK19 (fig. 4.6), ^{14}C-K28 (fig. 4.7), ^{14}C-T20 (fig. 4.8), ^{14}C-MAD 26 (fig. 4.9) and ^{14}C-G1 (fig. 4.10).

Variants A1-M1 of the ^{3}H-reference isolate, SK17, are marked (↓) on each fluorograph (a). Where different variants of these proteins occur in the ^{14}C-labelled isolate these are also indicated (▾).

Only the proteins of the ^{14}C-labelled isolates are detected on the direct autoradiographs (b) and the variants of proteins A-M present in each of these isolates are detailed (see also Table 4.2).

Fig. 4.5 ^{3}H SK17/^{14}C SK16

a)



b)

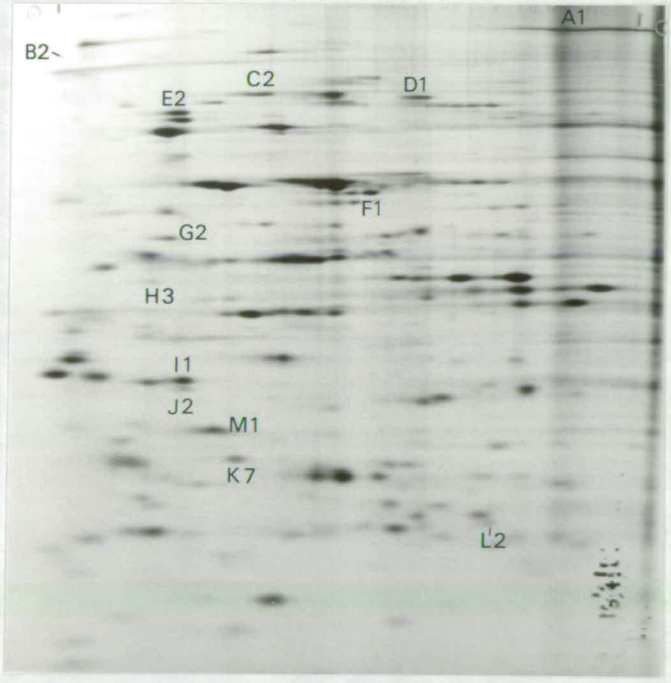


Fig. 4.6 ^{3}H SK17/^{14}C SK19

a)

b)

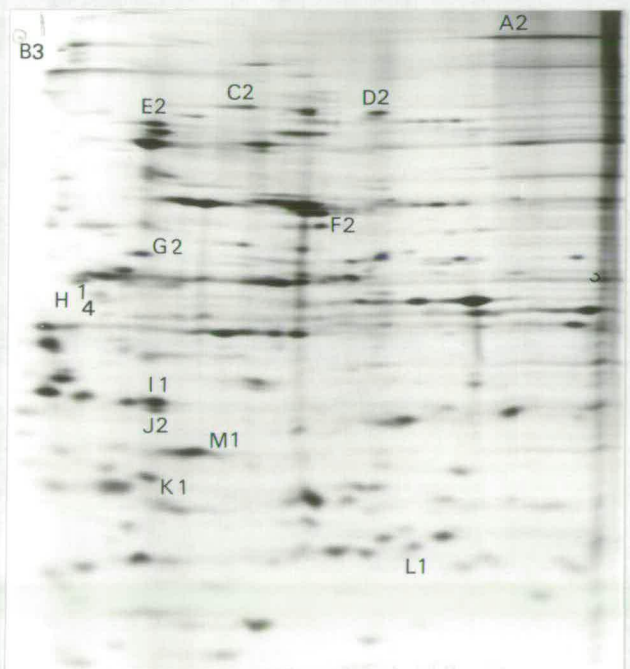
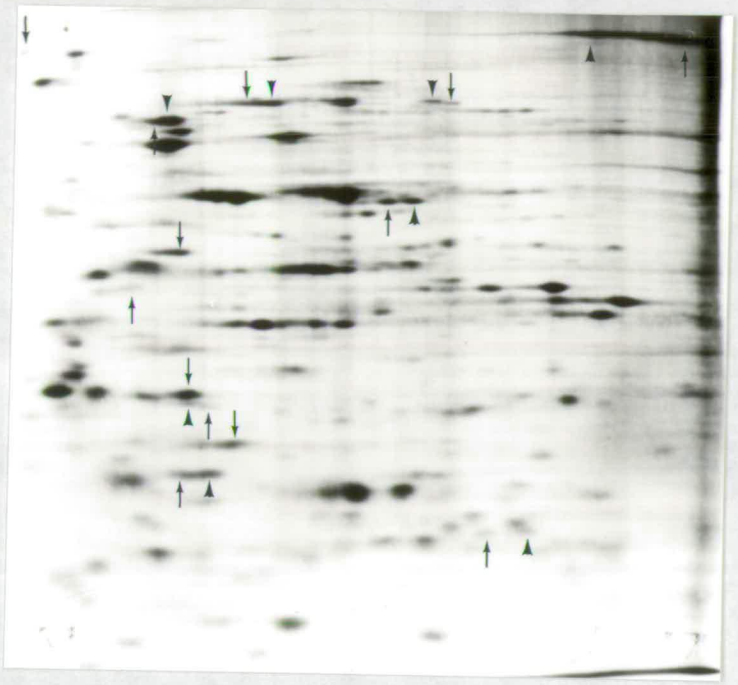


Fig. 4.7 ^{3}H SK17/^{14}C K28

a)



b)

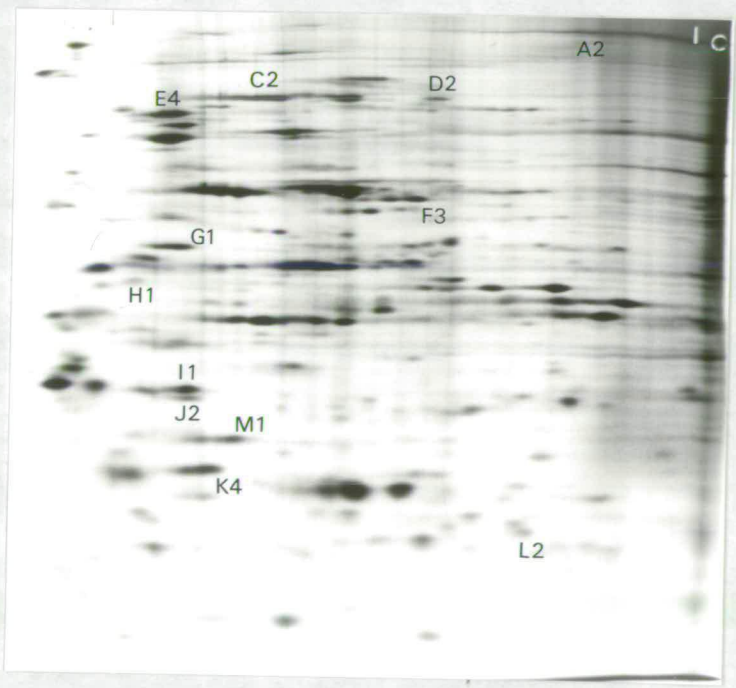
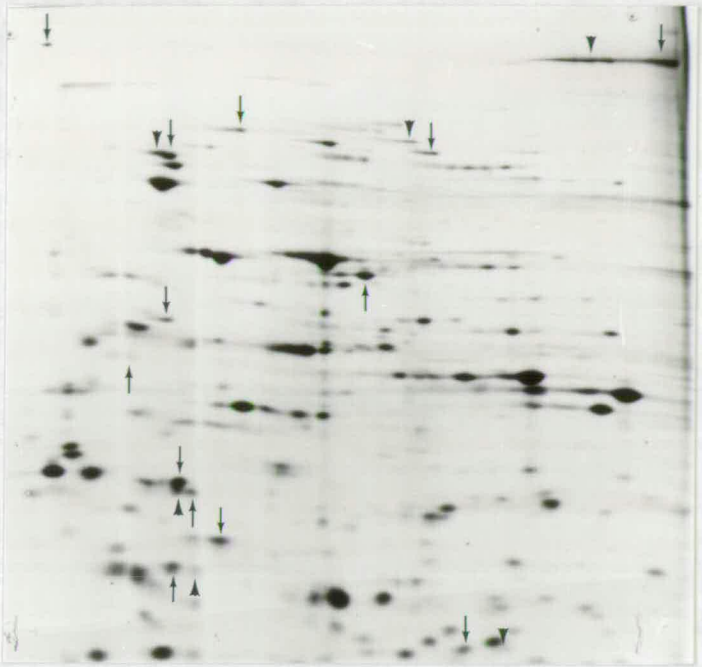


Fig. 4.8 ^{3}H SK17/^{14}C T20

a)



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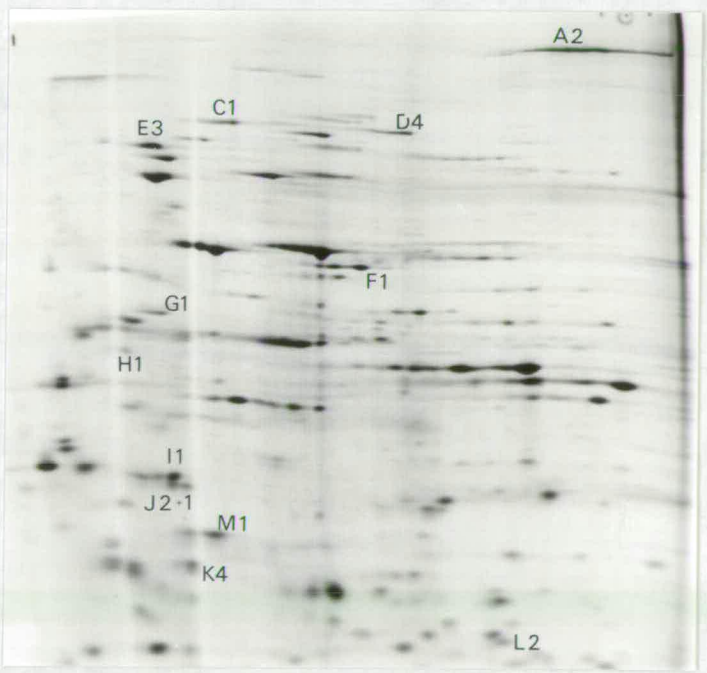
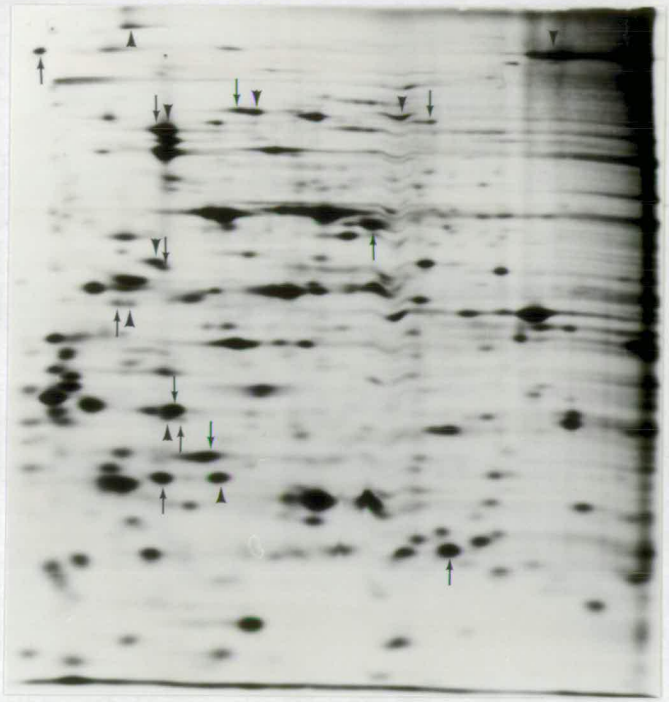


Fig. 4.9 ^{3}H SK17/^{14}C MAD 26

a)



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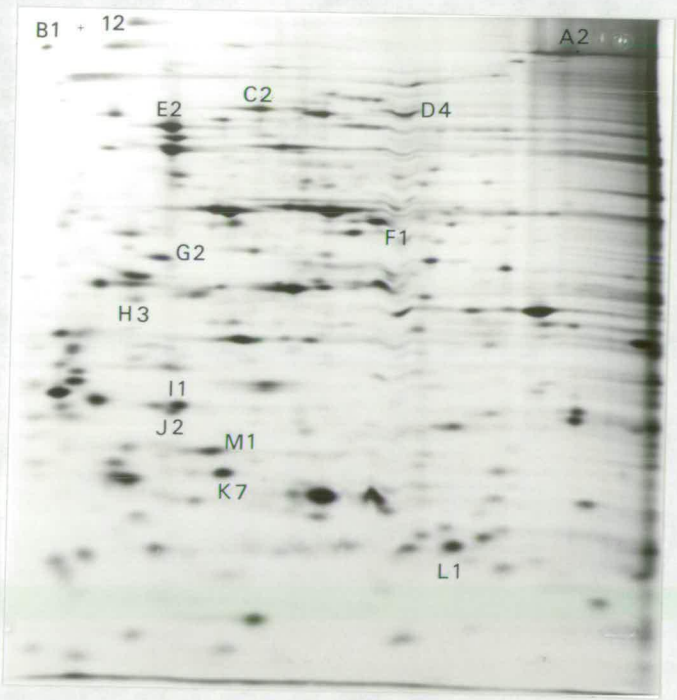
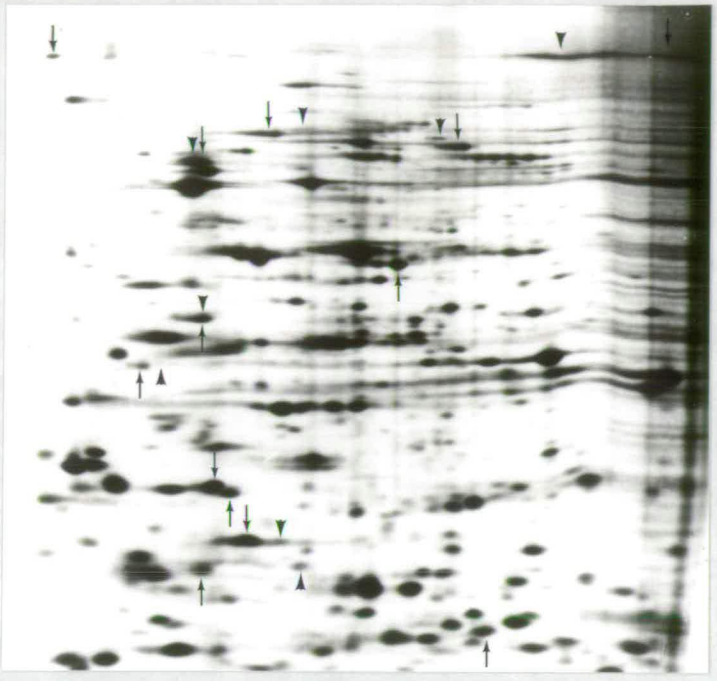
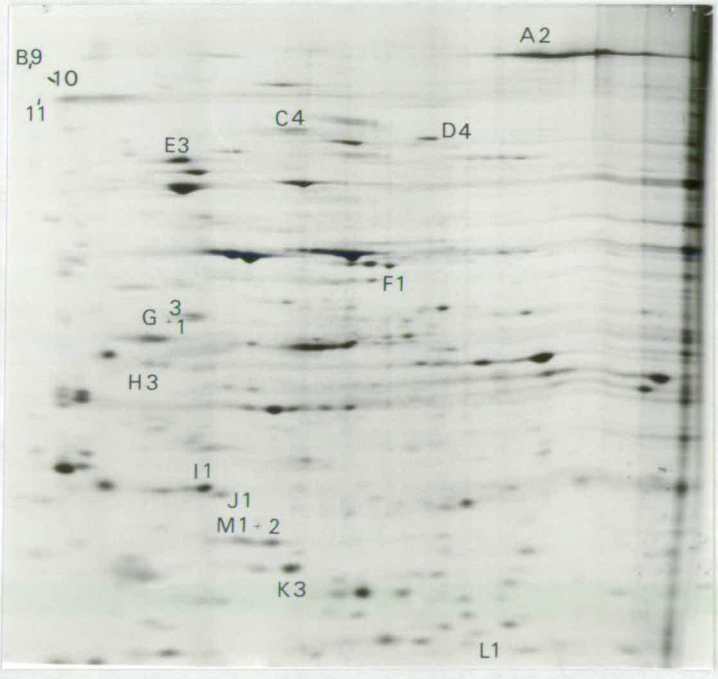


Fig. 4.10 ^{3}H SK17/^{14}C G1

a)



b)



of each protein. (A full account of these findings is given in Chapter 5). On the basis of this assumption, the data in Table 4.1 suggest that as many as fourteen of the twenty-one isolates may contain mixtures of parasites. However there are alternative explanations for the apparent occurrence of two variants of a protein. These reasons and their significance will be presented in section 4.6.

4.5 Geographical Variation

With a view to detecting any regional variation, the distribution of particular protein variants was examined among the isolates collected from Thailand, Papua New Guinea and the Gambia.

a) Variation within Thailand

The occurrence and distribution of protein variants among the Thai isolates fell into four categories:

i) One variant occurred in the majority of Thai isolates while other variants occurred only rarely, e.g. variants of proteins I and M,

ii) two or more variants occurred commonly in isolates from all regions of Thailand, e.g. proteins G and L,

iii) some variants were specific to, or more common in, isolates from certain regions. Variants A1 and D1, for example, were seen only in three isolates from Songhkla whereas variants A3, D5 and D6 were identified only in the Mae Sod (Tak) isolates,

iv) a more extreme pattern of variation is seen in protein B. This was so variable that only two pairs of isolates from the same

of each protein. (A full account of these findings is given in Chapter 5). On the basis of this assumption, the data in Table 4.1 suggest that as many as fourteen of the twenty-one isolates may contain mixtures of parasites. However there are alternative explanations for the apparent occurrence of two variants of a protein. These reasons and their significance will be presented in section 4.6.

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iv) a more extreme pattern of variation is seen in protein B. This was so variable that only two pairs of isolates from the same

region had variants of B in common. B2 was identified in both SK15 and SK16 and B3 in both SK18 and SK19. These pairs of isolates were also very similar in other respects.

The first two types of variation are indicative of a single interbreeding population of parasites within Thailand. However, the second two suggest that some degree of genetical isolation and regional differentiation may exist. It is of interest that the isolates from Mae Sod have a wider range of protein variants than isolates from the other two regions. The Songhkla isolates, for example, have variants 1 or 2 of protein D; the Kanchaburi isolates have D2, 3 or 4; while the Mae Sod isolates have D2, 3, 4, 5 or 6. A similar trend is seen for the variants of H and K. This greater degree of heterogeneity among the Mae Sod isolates - of which one (T17) was probably of Burmese and two (T19 and T22) of Cambodian origin - may in itself strengthen the case for regional variation within S. E. Asia.

b) Variation between Thai and Papua New Guinea isolates

There were many similarities between the six (identical) Papua New Guinean isolates and those from Thailand. The Papua New Guinean variants of ten of the thirteen variable proteins appeared commonly among the Thai isolates. However the variants of D and K were seen in only a minority of Thai isolates. Interestingly, variant B1 which had previously only been found in isolate SK17 appeared in these Papua New Guinea isolates.

c) Variation between Thai and Gambian isolates

The Gambian isolate, Gl, showed marked differences from the Thai isolates. Gl possessed unique variants of three proteins (B, C and G) and variants of four others (D, E, K and M) which were not common among the Thai isolates. Thus, over 50% of the variable proteins screened in Gl did not have 'typical' Thai variants. This suggests that major differences may exist between parasites from these two continents.

Similar trends can be seen in the pairwise comparisons of these isolates presented in fig. 4.4. Although the main impression that these numerical comparisons give is one of a high degree of intraspecific diversity, some regional differences can also be detected. Within Thailand, the Songhkla and Kanchanaburi isolates appear to show less intra-regional variation than do the Tak isolates and there is less inter-regional variation between Songhkla and Kanchanaburi than between either of these regions and Tak. However, again the most striking difference is between the Gambian isolate, Gl, and the Thai isolates. Gl has consistently fewer variants in common with the Thai isolates than they, on average, have amongst themselves.

The results presented above suggest that some degree of regional variation may exist within P. falciparum both between different regions of Thailand and between S.E. Asia and the Gambia. However it is obvious that much greater numbers of isolates, particularly of African origin, must be analysed in order to substantiate this finding.

4.6 Discussion

Protein variation in P. falciparum

The results presented in this chapter demonstrate the value of 2DGE for analysing variation in P. falciparum. Combining double-label autoradiography with the use of one isolate as a standard has considerably enhanced the ease and accuracy with which electrophoretic variation can be detected and has allowed strain comparisons to be based on a set of one hundred parasite proteins. 19% of the proteins screened were found to vary in one or more of the 21 isolates screened and 13 variant proteins were used for typing isolates (Table 4.2). Sufficient variation was found to differentiate between most of the isolates typed and the results presented here largely confirm those of Tait (1981). Both studies revealed a considerable amount of intraspecific variation in P. falciparum, described similar patterns of electophoretic variation in the proteins analysed and gave some indication that regional variation may occur in this species. However, there were some points of difference between the results presented here and those of Tait. For example, the percentage of proteins which varied between strains and the classification of certain proteins as variant or invariant differed. Thus, it is of interest to compare these two studies in more detail.

Tait (1981) analysed a set of 35 major parasite proteins in extracts of ^{35}S-methionine labelled parasite suspensions (see fig.

3.1). The identity of most of these proteins is detailed in Appendix 1. 14 (or 40%) of the 35 proteins were classed as varying electrophoretically in at least one of the seven isolates compared. The correspondence between Tait's variant proteins and those characterised in this thesis is shown in Table 4.3. Six of the 14 variant proteins correspond to proteins used for isolate typing in this chapter (proteins #1, 8, 36, 50 and 98) and a further three were observed to vary in certain isolates but were not among the set of major, reproducible parasite proteins defined in section 3.4. (#11, 26 and 135). Of the remaining variant proteins identified by Tait, one (AT #30) was not analysed here and four (proteins #19, 24, 53 and 81) were not found to vary in the isolates examined in this thesis. In the cases of proteins #24, 53 and 81 one variant predominated and a second occurred in only one of the seven isolates analysed by Tait. Therefore it is possible that no variation was detected in these proteins in this thesis simply because the rarer variant was not present in any of the isolates typed here. However, in the case of protein #19 Tait identified one variant in four isolates and a different variant in three others. Thus, some variation would have been expected among the 21 isolates screened in this chapter. An alternative explanation for the lack of variation is that the analysis of different single-labelled gels can be difficult and that the greater precision of the double-label system used here may have prevented misinterpretation of the 2D gel patterns of different strains.

In addition to reducing the likelihood of misclassifying invariant

Table 4.3 Correspondence between variant proteins described by Tait (1981) and those characterised in this thesis

Strain-variant proteins identified by Tait[i]			Corresponding proteins characterised in this thesis[ii]				
AT #	Type of variation	(nos. of variants)	#	Major, reproducible protein	Strain variation	Type of variation	(nos. of variants)
1	pI/MW[iii]	(6)	1 (B)	+	+	pI/MW	(10+)
3/4	pI/MW	(3)	8 (C)	+	+	pI/MW	(4)
5	pI/MW	(3)	11	-	+	pI/MW	(4)
7	pI/MW	(2)	19	+	-		
11	pI	(2)	24	-	-		
13	pI/MW	(3)	26	-	+	pI/MW	(4)
14	pI/MW	(2)	36 (G)	+	+	pI/MW	(2)
19	pI	(2)	53	+	-		
21	pI	(3)	50 (H)	+	+	pI/MW	(5)
29	pI	(2)	81	+	-		
30	pI	(2)	?				
31	pI	(2)	98 (M)	+	+	pI	(2)
34	pI	(2)	135?	+	+		

i) These proteins are identified in fig. 3.1. The pattern of variation and the number of different variants found among 7 isolates analysed is taken from Tait (1981).
ii) See fig. 3.11, Appendix 1 & 2 for further details of these proteins. (B) etc. refers to proteins used for strain typing.
iii) pI/MW : some, but not necessarily all, variants of a protein differ in both pI and MW.

proteins as variant, double-label analysis allows very small electrophoretic shifts to be detected with certainty. This partly explains why four variants of protein #13 were identified in this survey whereas no variation was observed by Tait.

Although both studies have several variant proteins in common it was not always possible to unambiguously determine which variants of a particular protein were equivalent in each study. Such comparisons were complicated by the use of different reference isolates and by the use of larger first and second dimension gels in the present work which slightly altered the pH profile and increased the resolving power of the system. However it is clear that patterns of electrophoretic variation similar to those described in section 4.3 also occurred amongst the variant proteins identified by Tait: some proteins (e.g. AT #31, 34) had variants which differed in pI only, others (e.g. AT #3, 4, 5 and 13) differed in both pI and MW and, finally, a "hypervariable" protein (AT #1) corresponding to protein #1 in this thesis had six electrophoretic variants of widely differing pI and MW among the seven isolates analysed.

As mentioned previously, Tait detected variation in 40% (14/35) of the proteins screened whereas only 19% varied here. This much lower proportion of variant proteins seems to be a direct effect of screening a larger number of proteins. If analysis had been confined to the set of proteins equivalent to those characterised by Tait then the estimated variation would have been about 32%. A large proportion of strain-variant proteins are among the 30-40

most intensely labelled (and most abundant) parasite proteins. By extending analysis to 100 proteins many more of the less abundant and relatively less variable proteins are included thereby reducing the estimate of the amount of intraspecific variation in P. falciparum.

Detection of isolates of mixed genotypes

In section 4.4,14 of the 21 isolates typed were identified as possible mixtures on the basis of having two variants of one or more proteins. However, there are at least three explanations for the simultaneous appearance of two variants:

i) the isolate is a mixture of parasites which have different variants of this protein,

ii) only a single genotype is present but two electrophoretic forms of the protein are produced, perhaps by partial modification of that protein,

iii) the chance appearance of an unrelated protein on the same region of the gel.

Isolates are most likely to be true mixtures when the two variants concerned usually occur independently in other isolates. This is the case for seven of the isolates:

K29 (L1 + 2); K36 (H1 + 3); T9 (A2 + 4, E2 + 3, F2 + 3, H3 + 6, K3 + 6); T17 (I1 + 2, K4 + 7); T20 (G1 + 2); T22 (A3 + 4, G1 + 2) and G1 (M1 + 2). However, in the case of SK19 (characterised by H1 + 4) and the Papua New Guinea isolates (B1 + 12), variants H4 and B12 have not been found independently in other isolates and their presence could be explained by ii) or iii) above.

The analysis of cloned lines derived from these isolates is needed to determine whether more than one type of parasite is present in these isolates. Taking the above considerations into account, a more realistic estimate of the number of mixed isolates among those screened would be seven out of 21 (K29, 36; T9, 17, 20, 22; G1). Tait also identified one such mixed isolate among the seven analysed and similar proportions of mixed infections have been detected by enzyme typing parasite isolates (Walliker, 1983a).

Geographical variation

Although only one Gambian isolate (G1) was analysed here, Tait (1981) compared two other Gambian isolates (FMG and G2) with five of S. E. Asian origin. Both studies detected considerable differences between the Gambian and S. E. Asian parasites. In section 4.5 it was noted that seven protein variants seen in G1 were either found rarely or not at all in Thai isolates. Similarly, in Tait's study, five proteins had variants which were either unique to the Gambian isolates or appeared rarely in the S. E. Asian isolates. Thus, if these two sets of results are considered together then they give a strong indication that the Gambian isolates do differ substantially from the geographically remote S. E. Asian parasites.

On a smaller geographical scale the results presented in section 4.5 show that some protein variants appeared to be specific to, or more common in, isolates from certain regions of Thailand suggesting that regional differences may also exist within S. E. Asia.

It is obvious that a much larger number of isolates must be

screened before any firm conclusions can be drawn about geographical variation in P. falciparum. However, the results presented here combined with those of Tait (1981) strongly suggest that such variation does occur and that two-dimensional gel analysis is a valuable technique for its investigation.

The results presented in this chapter are discussed more extensively in Chapter 9 in relation to

a) the variation detected in P. falciparum by other techniques such as enzyme electrophoresis and antigenic analysis, and

b) the protein variation observed in other experimental organisms by two-dimensional gel electrophoresis.

TWO-DIMENSIONAL GEL ANALYSIS OF A MIXED ISOLATE, T9, AND CLONED LINES DERIVED FROM IT

5.1 Introduction

It has been well documented that samples of malaria-infected blood, or cultures derived from them, are often heterogeneous for characters such as enzyme variants (Sanderson et al, 1981; Thaithong et al, 1981), antigens (Wilson et al, 1969; Hempelmann et al, 1981; McBride et al, 1982), proteins (Tait, 1981) and drug sensitivity (Trager et al, 1981; Thaithong, 1983). Therefore it is likely that an infected individual from an endemic area will harbour a mixture of genetically different parasites in his bloodstream. It is of considerable importance for basic research on malaria that such mixed isolates can be identified and that cloned lines can be produced. Cultures of single, defined parasite genotypes are of value for biochemical and immunological studies.

Techniques have successfully been developed to clone P. falciparum isolates by dilution methods (Rosario, 1981) or by micromanipulation (Trager et al, 1981) and the work presented here compares the two-dimensional gel protein patterns of one mixed isolate (T9) with those of several cloned lines derived from it. The T9 isolate was originally identified as a mixture of parasite genotypes by its possession of two electrophoretic forms of two different enzymes. This was subsequently proved by demonstrating that parasites of different enzyme types could be segregated by cloning experiments (Rosario, 1981). The uncloned isolate was

also shown to be heterogeneous for certain antigens (McBride, pers. comm.) and proteins (Table 4.2). The 2D gel analysis of T9 and clones derived from it was undertaken in order to characterise the cloned lines, to examine the heterogeneity within this isolate and to test the assumption that the presence of two variants of a protein in a single isolate implied that more than one genotype was present. Furthermore, since these clones had also been analysed for other characters, this study would allow the relative merits of different strain typing techniques to be assessed.

5.2 History of T9 and its Clones

The history of the T9 isolate and the method by which it was cloned are described by Rosario (1981). Immediately after being established in culture the isolate had two electrophoretic variants of the enzymes glucose phosphate isomerase (GPI-1 and GPI-2) and adenosine deaminase (ADA-1 and ADA-2). However over a period of a few weeks continuous culturing the enzyme variant ADA-2 disappeared from the culture and subsequently GPI-2 was also lost. This suggests that the isolate originally contained a minimum of three different parasite genotypes. At the time of cloning, variants GPI-1, GPI-2 and ADA-1 were present in the culture. One consequence of cloning by a dilution method is that a small proportion of the cultures produced will actually be mixtures and not clones. One such culture (1.1) produced by one cloning experiment was found to have both GPI enzyme types and this culture was

subjected to a further cloning. Some of the clones produced had GPI-1 only, some GPI-2 only and few had both enzyme types. Nine of these clones were later analysed by two-dimensional gel electrophoresis. As the mixed 'clone', 1.1, probably originated from only two T9 parasites, the cultures produced by the second round of cloning were likely to represent only two of the original T9 genotypes.

T9 and the cloned lines derived from it were cryopreserved for future study.

5.3 Characterisation of T9 and Clones

The uncloned T9 isolate, 1.1 and nine cloned cultures (c 13, 19, 23, 32, 34, 44, 57, 94, 96) were recovered from liquid nitrogen storage for two-dimensional gel analysis. 3-4 weeks after recovery the cultures were labelled with either the ^{3}H- or ^{14}C- labelled amino acid mixtures or with ^{35}S-methionine (section 2.2). The parasite extracts were then run on two-dimensional gels (section 2.4) and the proteins were analysed by single or double-label autoradiography (sections 2.6, 3.3).

For purposes of strain typing, the uncloned T9 (GPI-1 and GPI-2) and two representative clones, c94 (GPI-1) and c96 (GPI-2), were run on double-label gels with the SK17 isolate as a standard (fig. 5.1). The gels were analysed as detailed in Chapters 3 and 4. Seven clones (c 13, 19, 23, 32, 34, 94 and 96) were compared directly with uncloned T9 by double-label autoradiography. ^{14}C-labelled extracts of these clones were co-electrophoresed with

a)

b)

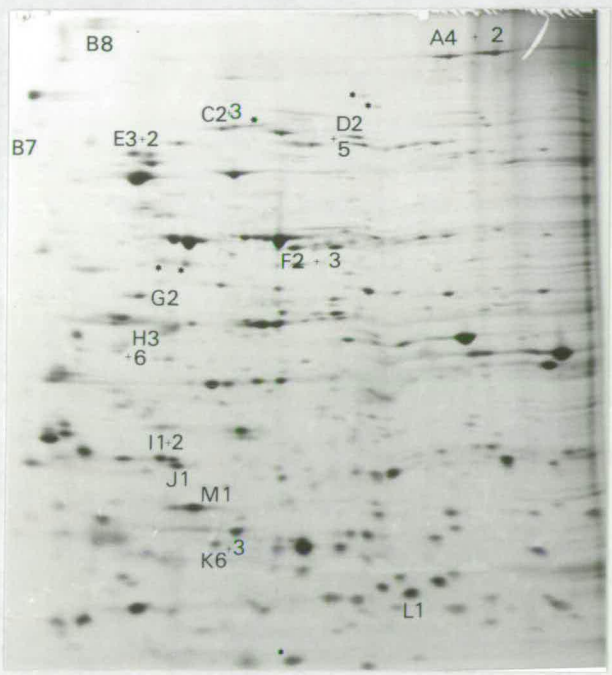


Fig. 5.1 a) is the fluorograph of a double-label gel of ^{3}H-SK17/^{14}C-T9 and b) is the direct autoradiograph of the same gel (i. e. showing only the ^{14}C-labelled proteins of T9). Variants of proteins A-M used for strain typing are marked: ↓ denotes those specific to SK17 and ▼ those specific to T9. * indicates other proteins which vary reproducibly between SK17 and T9.

a)

b)

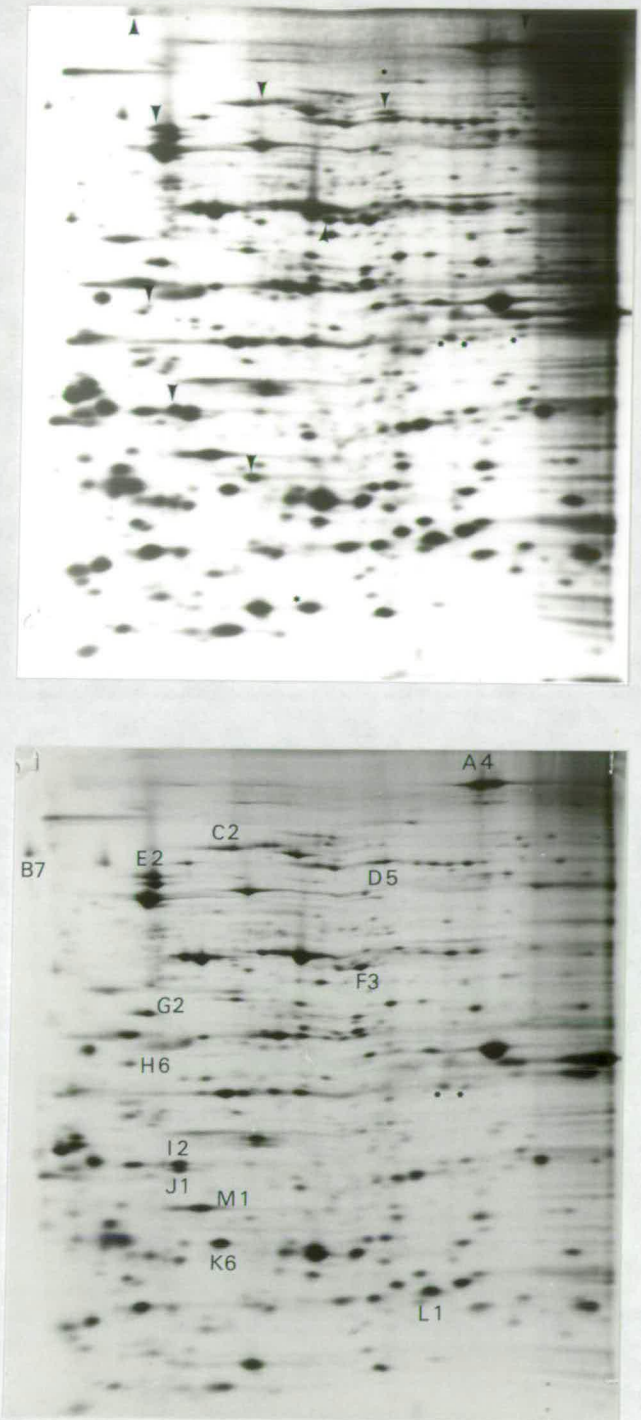
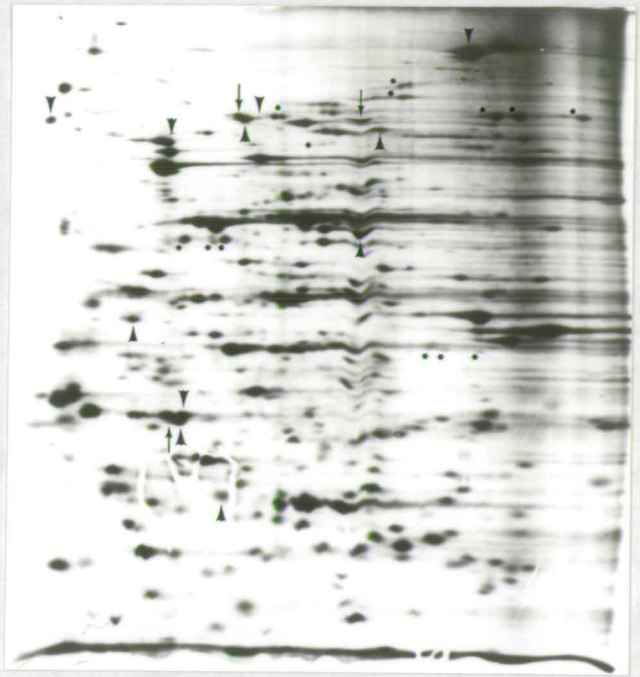


Fig. 5.2 The fluorograph (a) and the direct autoradiograph (b) of a gel of ^{3}H-T9/^{14}C-c32. Variants of proteins A-M are shown and those present only in uncloned T9 are indicated (▾). * Marks other proteins seen only in the uncloned isolate.

a)



b)

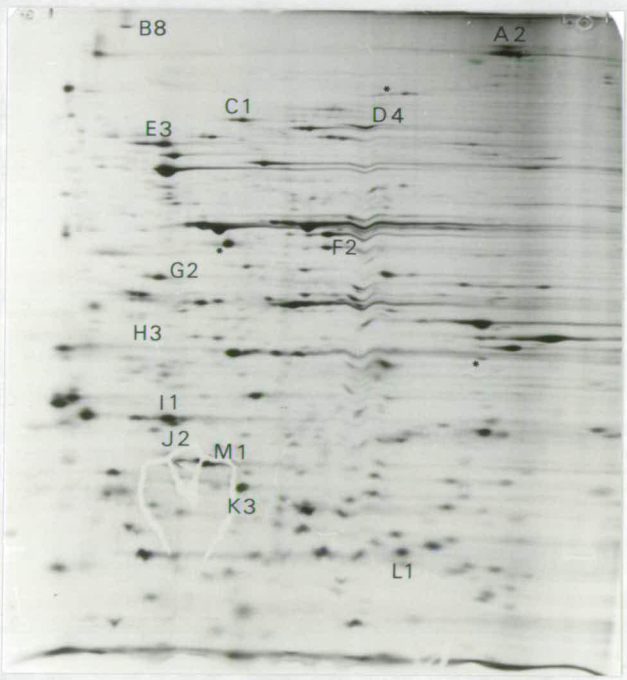


Fig. 5.3 The fluorograph (a) and direct autoradiograph (b) of a gel of ^{3}H-T9/^{14}C-c19. The variants of proteins A-M are marked: ▾ indicates those present only in T9 and ↓ those specific to c19. * identifies other proteins which differ between the two cultures.

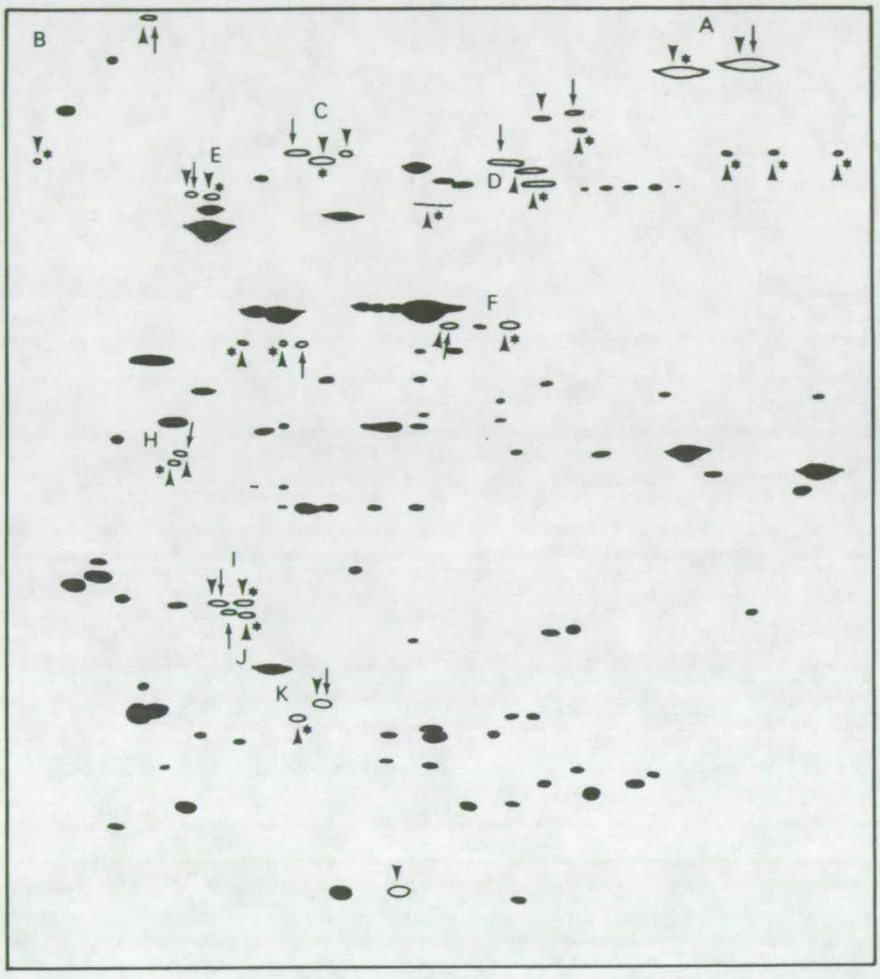


Fig. 5.4 Diagram summarising the variation detected in proteins A-M used for strain typing and other reproducible differences observed in the uncloned T9 isolate and cloned lines derived from it (see figs. 5.1-5.3). Variant proteins are shown as open spots: ▾ indicates those detected in uncloned T9; * indicates those present in c32-type parasites (genotype A, Table 5.2); and ↓ those found in c19-type cultures (genotype B, Table 5.2).

^{3}H-labelled proteins of the uncloned isolate. Some of these autoradiographs are illustrated in figs. 5.2-5.3. Following characterisation by the above methods further screening of T9, 1.1 and the remaining clones (c 44 and c 57) was performed by running single-label gels of parasites labelled with ^{35}S-methionine or ^{14}C-amino acids. (Figs. 5.6-5.7).

Initially, T9 and its clones were typed by means of the set of variable proteins established for isolate-typing in Chapter 4 (see fig. 4.1). The results of this analysis are presented in Table 5.1. Two classes of clones were found with c 32 and c 94 falling into one category and c 13, 19, 23, 57 and 96 into the other. In addition clones 34 and 44 appeared to be mixtures since some of the c 96-type variants were detected in addition to those of the c 94 types.

The two classes of clone (compare c 94 and c 96 in Table 5.1) differed considerably from each other and had only 3 protein variants in common out of the 13 analysed. With the exception of c 34 and 44, all the clones had only a single variant of each protein whereas uncloned T9 had two variants of each of 9 proteins. In the case of 7 of the variable proteins (A, B, E, F, H, I and K) where the uncloned isolate had 2 variants, the c 94-type clones had one of these variants and the c 96-types had the other. For example, T9 had both A2 and A4 whereas c 94 had A4 only and c 96 A2 only. This was the type of distribution of variants which was expected to appear amongst the cloned lines. However, the variants of 3 proteins (C, D and J) did not conform to this pattern. In each case the c 96-types

Table 5.1 The protein variants of T9 and its clones

Culture	Variant proteins												
	A	B	C	D	E	F	G	H	I	J	K	L	M
T9	2,4(i)	7,8	2,3	2,5	2,3	2,3	2	3,6	1,2	1	3,6	1	1
1.1	4 >2	7	2	5	2	3	2	6	2	1	6 >3	1	1
c13	2	8	1	4	3	2	2	3	1	2	3	1	1
c19	2	8	1	4	3	2	2	3	1	2	3	1	1
c23	2	8	1	4	3	2	2	3	1	2	3	1	1
c32	4	7	2	5	2	3	2	6	2	1	6	1	1
c34	4	7	2	5	2	2,3	2	6	2	1	6 >3	1	1
c44	4 >2	?	2	5	2	3	2	6	2	1	6 >3	1	1
c57	2	8	1	4	3	2	2	3	1	2	3	1	1
c94	4	7	2	5	2	3	2	6	2	1	6	1	1
c96	2	8	1	4	3	2	2	3	1	2	3	1	1

(i) variants were of approximately equal intensity unless indicated (>).

had a variant which did not appear on gels of the uncloned isolate e.g. C2 and C3 were found in uncloned T9, C2 in c 94 but C1 in c 96. Also the uncloned T9 had variants C3 and D2 which were not identified in either of the two types of clone.

The most probable interpretation of these findings is that the c 96 genotype had largely or completely disappeared from the uncloned T9 culture sometime after the cloning was performed. In addition T9 must contain one or more parasite genotypes which did not appear in the clones analysed here.

On the basis of this interpretation, analysis of the mixed 'clone' 1.1, was expected to show a mixture of the c 94 and c 96 genotypes. However, this could not be proved conclusively since at the time of labelling the c 94 genotype was predominant and only two variable proteins (A and K) had the expected pair of variants.

The proposed explanation of the origin of the T9 clones is as follows (fig. 5.5): At the time of cloning, T9 contained a minimum of three different genotypes of parasite - A, B and C. B was lost during subsequent culturing leaving only A and C at the time of two-dimensional gel analysis. Genotypes A and B were present in the mixed 'clone', 1.1, and these were then separated out by the second round of cloning. The variant proteins of the A, B and putative C genotypes of parasite are given in Table 5.2.

In addition to the variation detected among the standard set of variable proteins other reproducible differences were observed between the two types of clone and the uncloned T9. These are

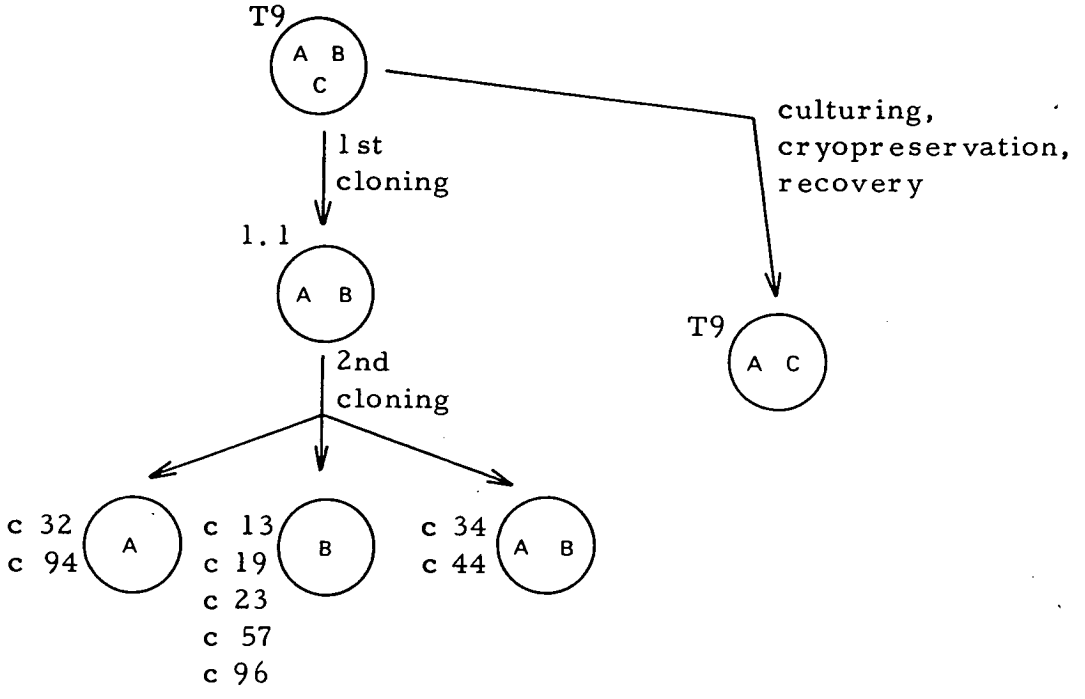


Fig. 5.5 Proposed derivation of the T9 clones from an original mixture of the three parasite genotypes (A, B and C) defined in Table 5.2.

detailed in figs. 5.1-5.3 and shown diagramatically in fig. 5.4. In all instances, this extra variation confirmed the previous conclusion that only two different parasite genotypes were present in the cultures produced by the second cloning. Further analysis of these differences also supported the proposed origin of the clones from the uncloned T9 as detailed above.

5.4 Changes in Labelling Pattern during Continuous Culture

The T9 culture and five clones (c 13, 32, 34, 94 and 96) were

Table 5.2 Summary of the protein variants of T9 and its component genotypes

Culture	Genotype	GPI type(i)	Variant proteins A	B	C	D	E	F	G	H	I	J	K	L	M
T9	A+C	1+2	2,4	7,8	2,3	2,5	2,3	2,3	2	3,6	1,2	1	3,6	1	1
c32,94	A	1	4	7	2	5	2	3	2	6	2	1	6	1	1
c13,19,23, 57,96	B	2	2	8	1	4	3	2	2	3	1	2	3	1	1
-	C(ii)	2	2	8	3	2	3	2	2	3	1	1	3	1	1

(i) Walliker (pers. comm.) and Thaithong et al (1984).
(ii) Genotype C was not present in any of the clones analysed. Its protein variants have been deduced from comparison of the c94-type variants with those present in uncloned T9.

labelled and re-analysed after a further 5-6 weeks in culture. The results are presented in Table 5.3. Autoradiographs of the uncloned T9 culture labelled 4 and 10 weeks after recovery from cryopreservation are shown in figs. 5.1 and 5.7 respectively.

In the uncloned isolate the results show that genotype A (the c 94 type) had become relatively more abundant and genotype C less abundant during the six week period in culture. For example, A2 was now much reduced in intensity of labelling relative to variant A4 and the same was observed for the genotype C variants of proteins D, F, H and K. In the cases of proteins B, C, E and I the variants attributed to genotype C could no longer be detected on the autoradiographs. Thus, genotype A (c 94) parasites were gradually outgrowing the other parasites in the T9 culture.

Of the clones which were re-analysed, c 32 and c 94 had protein compositions identical to those observed six weeks earlier. Obviously this was the expected outcome for cultures which were true clones. However, two cultures - c 13 and c 96 - which had previously appeared to be genuine clones appeared to be mixtures of genotypes A (c 94) and B (c 96) after an interval of 6 weeks. Again it seemed that genotype A was gradually outgrowing the other parasites in these cultures. This finding could not be explained by c 13 and c 96 originally being mixtures of genotypes A and B otherwise the genotype A variants would have been detectable after 4 weeks in culture rather than 10. However, a possible explanation is that c 13 and c 96 were initially good clones but were accidentally

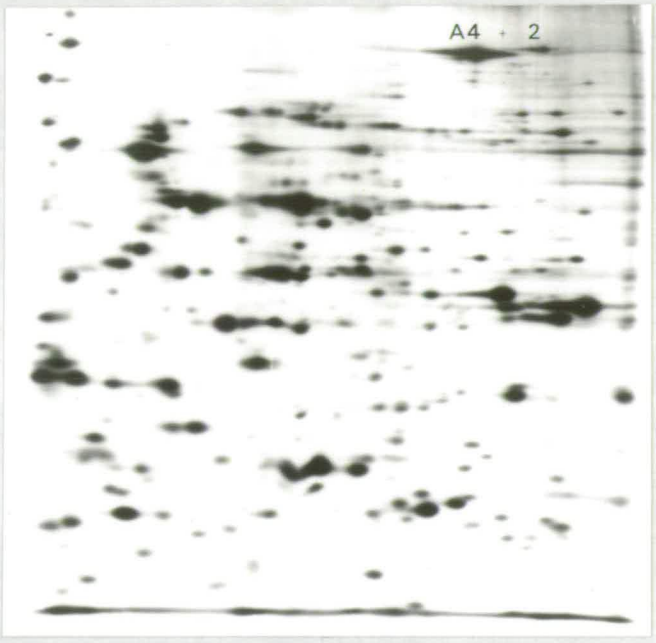


Fig. 5.6 2D gel of ^{14}C-labelled 'clone' 34 showing two variants of protein A.

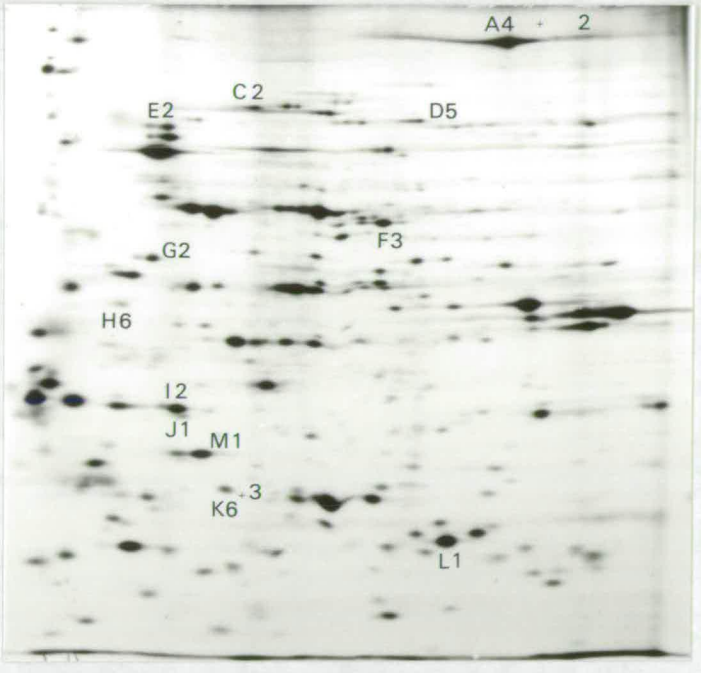


Fig. 5.7 Uncloned T9 labelled with ^{14}C-amino acids 10 weeks after recovery of cryopreserved parasites. (cf. fig. 5.1 b of T9 labelled after 4 weeks). The c32-type (genotype A) variants predominate.

Table 5.3 Changes in the protein composition of cloned and uncloned T9 over a period of six weeks in culture

Culture	Week(i)	Variant proteins												
		A	B	C	D	E	F	G	H	I	J	K	L	M
T9	4	2=4(ii)	7=8	2=3	2=5	2=3	2=3	2	3=6	1=2	1	3=6	1	1
	10	4≫2	?	2	2≫5	2	3≫2	2	6	2≫1	1	6≫3	1	1
c32,c94	4	4	7	2	5	2	3	2	6	2	1	6	1	1
	10	4	7	2	5	2	3	2	6	2	1	6	1	1
c34	4	4	7	2	5	2	2=3	2	6	2	1	6≫3	1	1
	10	4≫2	?	2	5	2≫3	3	2	6	2	1	6≫3	1	1
c13	4	2	8	1	4	3	2	2	3	1	2	3	1	1
	10	2>4	8	1>2	4>5	3>2	2>3	2	3	1>2	2>1	3>6	1	1
c96	4	2	8	1	4	3	2	2	3	1	2	3	1	1
	10	4>2	?	2	5	2>3	3	2	6	2	1	3=6	1	1

(i) Number of weeks after recovery of cryopreserved material.
(ii) =, >, ≫ indicate the relative intensities of labelling where two variants occur in one culture.

contaminated with genotype A (c 94) parasites some time after the cloning. The faster growing genotype A parasites would then have slowly increased in relative abundance and eventually their protein variants would be detectable on the two-dimensional gels. This hypothesis of accidental contamination was supported by the fact that a culture of c 96 maintained independantly in Thailand was found to contain only the c 96 (or genotype B) variants even after several weeks in culture.

5.5 Discussion

One practical aspect of the results presented in this chapter is their significance for the analysis and interpretation of the protein patterns seen on 2D gels. Whereas 2D gel analysis of the uncloned T9 identified two putative variants of each of nine different proteins, clones derived from T9 had only a single variant of each protein. This supports the interpretation that the presence of double variants on 2D gels is a result of the presence of two or more parasite genotypes in the culture analysed. (See sections 4.4 and 4.6).

In addition to 2D gel analysis, parallel studies were carried out on T9 and its clones to determine their enzyme types and drug sensitivities (Walliker, pers. comm.; Thaithong, 1983) and to characterise them antigenically using strain-specific monoclonal antibodies (McBride, pers. comm.) Study of all the above characters supported the conclusion drawn from the two-dimensional gel analysis of the proteins that only two types of parasite appeared

among these T9 clones. In vitro drug tests detected two levels of sensitivity to chloroquine with the genotype A parasites being less sensitive than those of genotype B. The enzyme typing data for GPI showed that cultures of genotype A (e.g. c 94) had GPI-1, those of genotype B (e.g. c 96) had GPI-2 and those which were mixtures (e.g. 1-1, c 34, c 44) had both GPI-1 and GPI-2. Uncloned T9 also had both GPI variants which implied that the proposed genotype C would be characterised by GPI-2. Antigenic analysis also identified two types of parasite among the clones. The uncloned T9 had two major antigenic types only one of which appeared in some clones (e.g. c 94, genotype A). A third minor component was also detected and this was equivalent to that found in the other class of clone (e.g. c 96, genotype B).

Both enzyme typing and antigenic analysis detected the changes in the composition of certain 'clones' during in vitro culture (as described in section 5.3). They also confirmed that one type of parasite (genotype A) consistently outgrew the other components of T9.

The various techniques applied to the characterisation of the T9 clones have different relative merits which are discussed in Chapter 9. However, the most striking difference is the greater sensitivity of the antigenic analysis. It has been estimated (McBride, pers. comm.) that an antigenic type making up as little as 0.1% of a parasite population may be detected by immunofluorescence whereas it must make up about 5% of the population to be detectable by enzyme typing or two-dimensional gel analysis. This higher sensitivity allowed the

immunofluorescence technique to detect a small proportion of the third antigenic type of parasite (equivalent to the genotype B of c 96) in the uncloned T9. This genotype subsequently disappeared from the culture. Thus, this provides support for the presence of 3 (or more) genotypes in T9 at the time of cloning and for the validity of the scheme presented in fig. 5. 5.

As explained in section 5. 2 only two parasite genotypes were expected to be found among these clones. However the results show that at least three different genotypes must have been present in T9 at the time of cloning. One enzyme type (ADA-2) was lost prior to cloning, therefore it can be surmised that when originally isolated T9 must have contained a minimum of four different parasite genotypes. In fact, the T9 isolate has since been cloned again and, among the clones analysed, five different genotypes including the two described here have been characterised (Thaithong et al, 1984; Fenton et al, in preparation). The clones were compared for enzyme types, antigens, sensitivity to chloroquine and pyrimethamine, presence of knobs and 2D protein variants. One practical result of this intensive analysis is that a set of distinct and well characterised cloned lines are available for basic research on P. falciparum. Furthermore, the results suggest that a large amount of genetic diversity may exist among the parasites present in a single sample of infected blood.

STAGE-SPECIFIC PROTEIN SYNTHESIS

6.1 Introduction

During the development of the intraerythrocytic parasite many events occur. The parasite increases both in size and in complexity of organisation as it progresses from the ring form to the mature schizont. Changes are induced in the infected cell's metabolism and in the structure, properties and morphology of the erythrocytic membrane. The stage-dependent synthesis of parasite proteins is therefore of fundamental importance to the cell biology of Plasmodium. It is also of practical relevance. In genetic engineering, for example, information about the time of synthesis of specific proteins is of value if mRNA species are to be used for cloning parasite genes or screening gene libraries.

Although P. falciparum parasites develop synchronously in natural infections this synchrony is lost when isolates are adapted to in vitro culture conditions and until recently this has limited the study of stage-specific events. However, in 1978 Pasvol et al reported that erythrocytes infected with schizonts could be isolated by their ability to remain suspended in gelatin solutions. In addition, Lambros and Vanderberg (1979) demonstrated that incubation with sorbitol could preferentially lyse cells containing mature parasites. Thus, these techniques made it possible either to selectively concentrate or to selectively kill erythrocytes infected with mature parasites. Highly synchronous cultures can be produced in vitro by

applying these procedures either alone (Lambros and Vanderberg, 1979; Myler et al, 1982) or in combination (Kilejian, 1980a; Perkins, 1982). Other ways of synchronising development have also been described. These include density gradient centrifugation to physically separate cells infected with different parasite stages (Mrema et al, 1979; Kramer et al, 1982) and the use of chemicals, e.g. colchicine (Hui et al, 1983), to induce synchrony.

One-dimensional gel analysis of stage-specific protein synthesis has been reported previously for P. falciparum (e.g. Kilejian, 1980a; Perrin and Dayal, 1982; Allred and Sherman, 1983; Deans et al, 1983b; Myler, Saul and Kidson, 1983) and for other species of Plasmodium (Newbold et al, 1982a; Deans et al, 1983a; Sherman and Tanigoshi, 1983). The main aims of the work presented here were to apply two-dimensional gel electrophoresis to the study of stage-specific proteins and to characterise the set of major, reproducible proteins of P. falciparum (defined in section 3.4) in terms of their pattern of synthesis during the asexual cycle. An additional objective was to identify any specific parasite proteins which may be present in the culture medium or lost during the preparation of parasite extracts of synchronous cultures. Clones of the Thai isolate T9 which had already been extensively analysed by 2DGE (Chapter 5) were chosen for this study in preference to potentially mixed parasite populations of uncloned isolates. Representative cultures of the two genotypes found amongst these clones (A - clones 32 and 94; B - clone 96) were used in the experiments described below.

6.2 Synchronisation and Metabolic Labelling

T9 clones 32 and 94 were synchronised by repeated sorbitol treatments as described in detail in section 2.1.4. Successive rounds of sorbitol lysis were carried out over several growth cycles - usually more than five - until the developmental age difference of parasites in the culture was less than eight hours. Clone 96 was either synchronised by sorbitol treatments alone or by the combination of sorbitol lysis and flotation in Plasmagel described in section 2.1.4. The latter technique could only be applied to c 96 because neither c 32 or c 94 produced schizont-infected erythrocytes which could be concentrated by flotation in gelatin solutions. As with the sorbitol procedure, the Plasmagel/sorbitol treatments were repeated over several growth cycles until the necessary degree of synchrony and yield of parasites was produced. Fig. 6.1 shows Giemsa-stained blood smears of successive stages of a synchronised culture of clone 32.

Parasite proteins were metabolically labelled by incubating the cultures at 2-5% parasitaemia with 10-50 μCi/ml ^{35}S-methionine as described in section 2.2. Three periods of labelling were chosen to correspond approximately to the development of rings, trophozoites and schizonts respectively. Cultures of rings were labelled shortly after the final sorbitol treatment for up to 15 hours. Initially the parasites were approximately 1-9 hours old (i.e. 1-9 hours post-invasion) and at the end of the labelling period they were mainly late

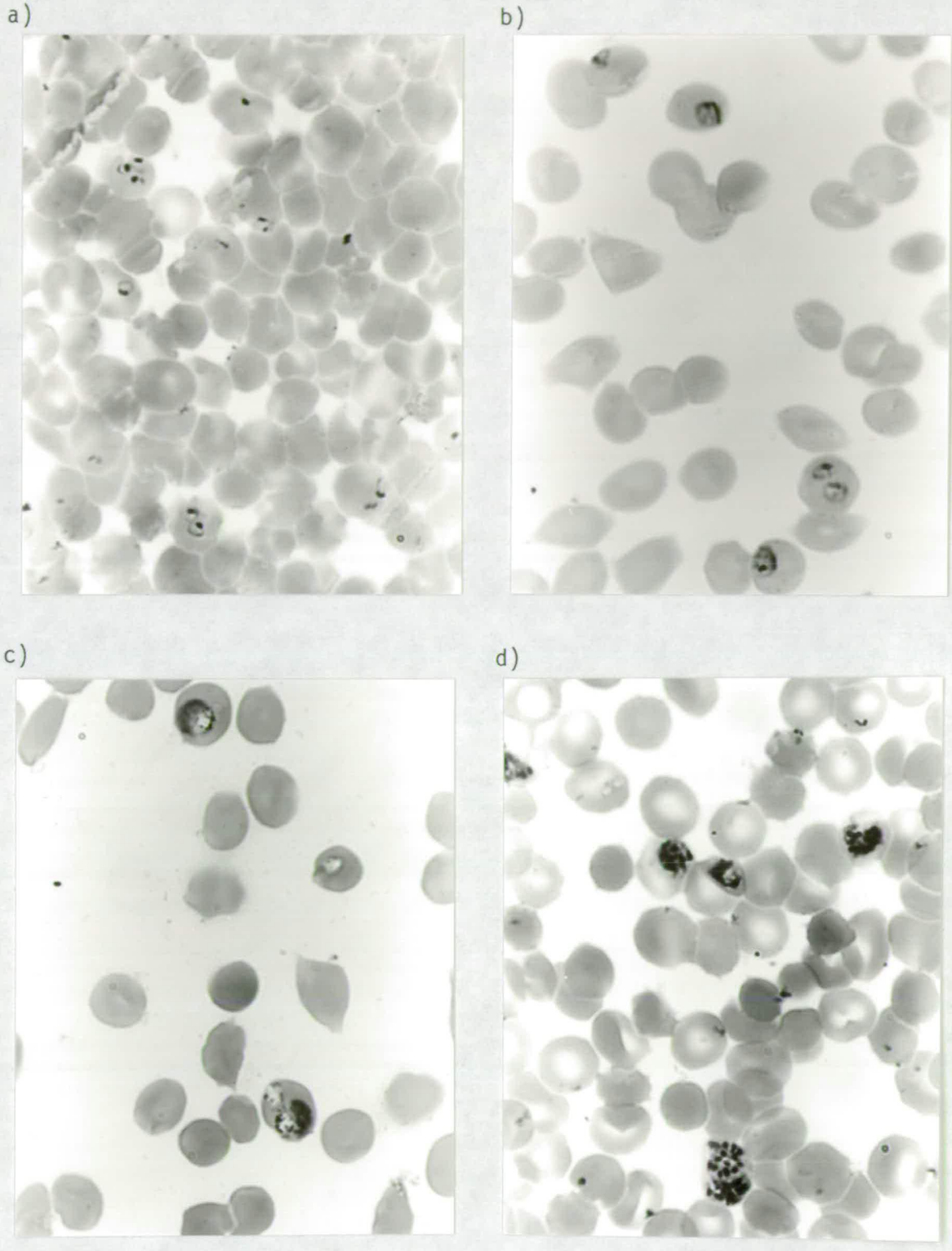
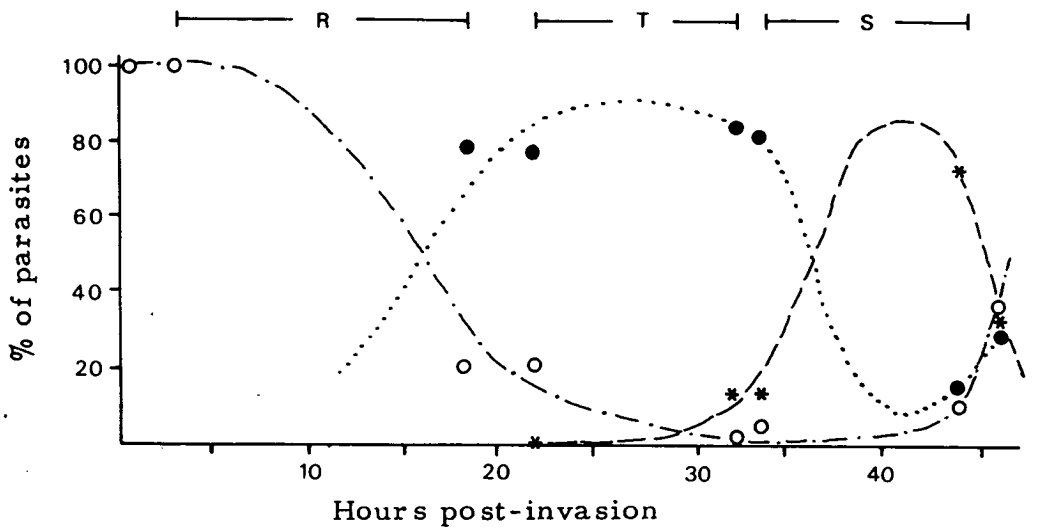


Fig. 6.1 Giemsa-stained smears of a synchronous culture of T9 c32 approximately 1 (a), 21 (b), 29 (c) and 41 (d) hours after sorbitol treatment showing young rings, early trophozoites, late trophozoites and segmenting schizonts respectively.

ring and early trophozoite stages. A second labelling period of up to 10 hours was initiated when the parasites were 20-28 hours old and the harvested cultures consisted predominantly of mature trophozoites and some schizonts. The final labelling period covered the stage of nuclear division commencing when the parasites were 30-38 hours old and continuing for about 10 hours. Some merozoite release and invasion had usually occurred by the end of this period but contributed little to the total incorporation of label. Details of the exact labelling periods used and of the corresponding compositions of two synchronous cultures are shown in fig. 6.2. Parasites were prepared from labelled cultures by saponin lysis (section 2.1.5) and the resulting pellets were solubilised for one- or two-dimensional gel electrophoresis as appropriate (section 2.3). In one experiment, labelled cultures were separated into three fractions: culture medium, material lost during saponin-lysis and saponin-released parasites. The culture medium was collected after centrifuging the labelled culture at 1500 g for 10 minutes at 4°C. The sedimented erythrocytes were lysed at 4°C with saponin and, after centrifugation at 2500 g for 15 minutes at 4°C, the red cell ghost layer and supernatant were collected, mixed and designated as 'saponin supernatant'. The remaining pellet of released parasites was washed twice with RPMI prior to solubilisation. Both the culture medium and saponin supernatant were dialysed against phosphate-buffered saline containing 1 mM PMSF and then solubilised for gel electrophoresis (section 2.3).

Samples of solubilised, labelled extracts were processed for

T9c32



T9c96

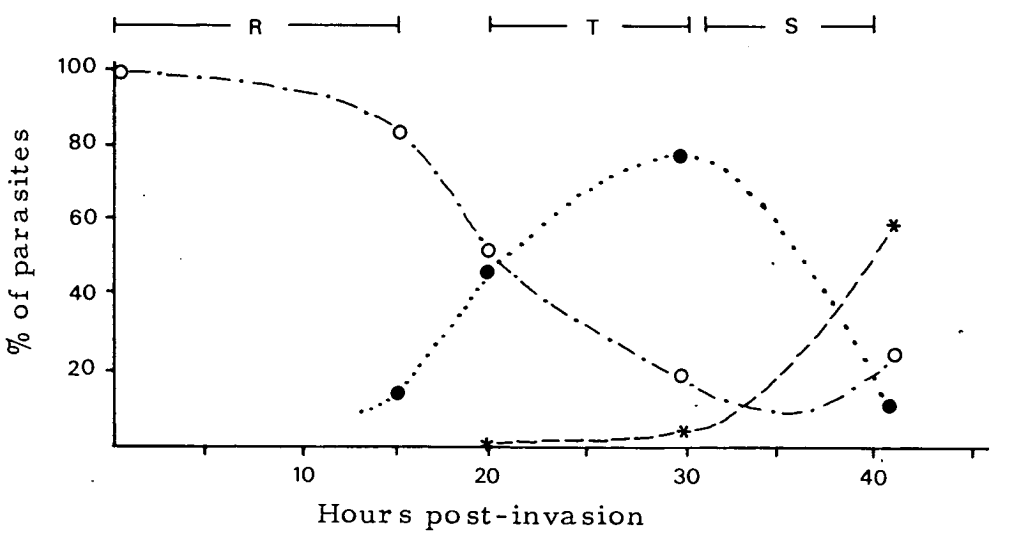


Fig. 6.2 The percentage of rings (-·—o—·—-), trophozoites (·····●·····) and schizonts (– – –*– – –) in synchronous cultures of T9 c32 and c96 over the course of two labelling experiments. The three labelling periods used are shown (⊢— —⊣) for each culture.

liquid scintillation counting as described in section 2.3 to determine the amount of ^{35}S-methionine incorporated into TCA-precipitable material. It was found that the amount of label incorporated by a given number of parasites and recovered in solubilised extracts tended to increase with the developmental age of parasites in the synchronised cultures. This is illustrated in fig. 6.3 (a) where the levels of incorporation have been expressed as the percentage of label incorporated per 10^7 parasites over a ten hour labelling period. This result simply shows that the larger, more complex parasites synthesise more protein than do smaller, younger stages and does not imply anything about the true specific activity of labelling, i.e. cpm incorporated per unit parasite protein. It is of interest that an asynchronous culture, labelled under almost identical conditions as used for the synchronous culture of the same clone (32) gave a similar, low level of incorporation to the ring stage parasites.

Estimates were also made of the relative amounts of TCA-precipitable material recovered in solubilised preparations of parasites, saponin supernatants and culture media from both synchronous and asynchronous cultures of clone 32 (fig. 6.3 (b)). The cpm incorporated by synchronised cultures into the three fractions tended to increase with the developmental age of the parasites and in all cases a large proportion of the total TCA-precipitable cpm was recovered in the culture medium (20-35%) and saponin-supernatant (25-60%). This was also true for the asynchronous culture in which an estimated 50% was present in the culture medium and 37% in the saponin supernatant.

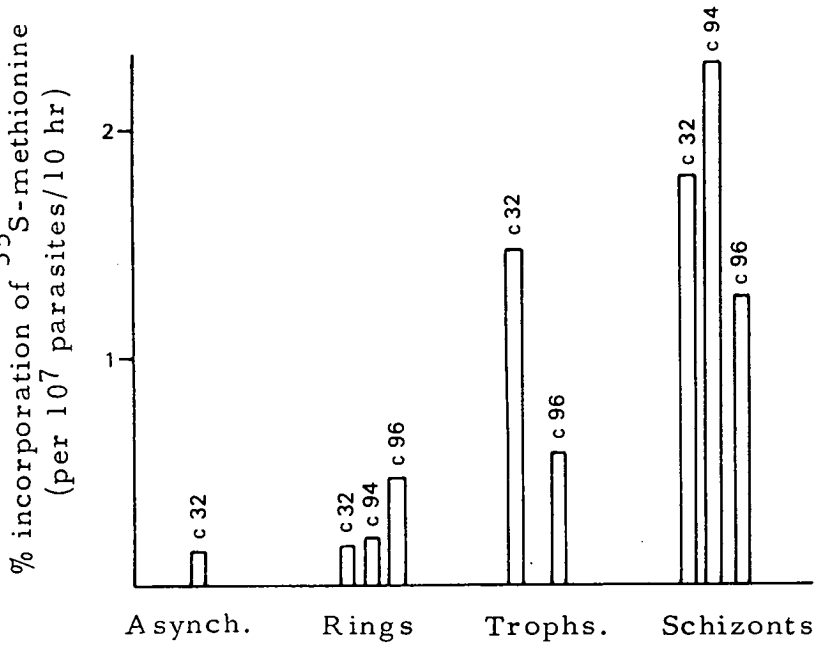


Fig. 6.3 a) The incorporation of ^{35}S-methionine into TCA-precipitable material recovered in saponin-released parasites from synchronous and asynchronous cultures of T9 clones 32, 94 and 96. To compare the results of different labelling experiments the incorporation was estimated per 10^7 parasites over a 10 hour labelling period.

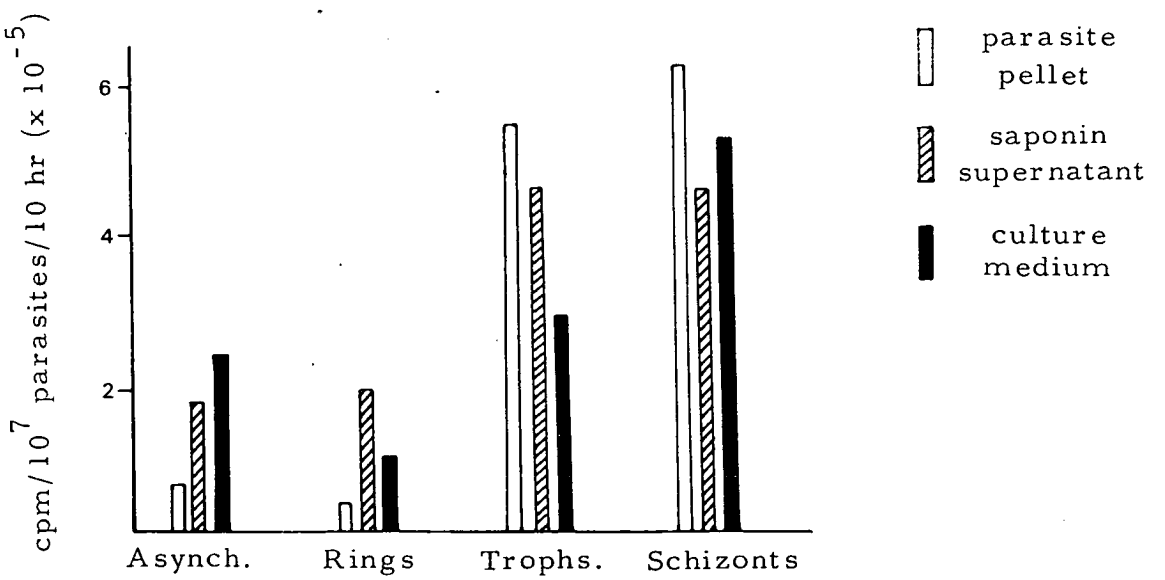


Fig. 6.3 b) The recovery of TCA-precipitable cpm in parasite pellets, saponin supernatants and culture medium from asynchronous, ring, trophozoite and schizont-stage cultures of T9 c32.

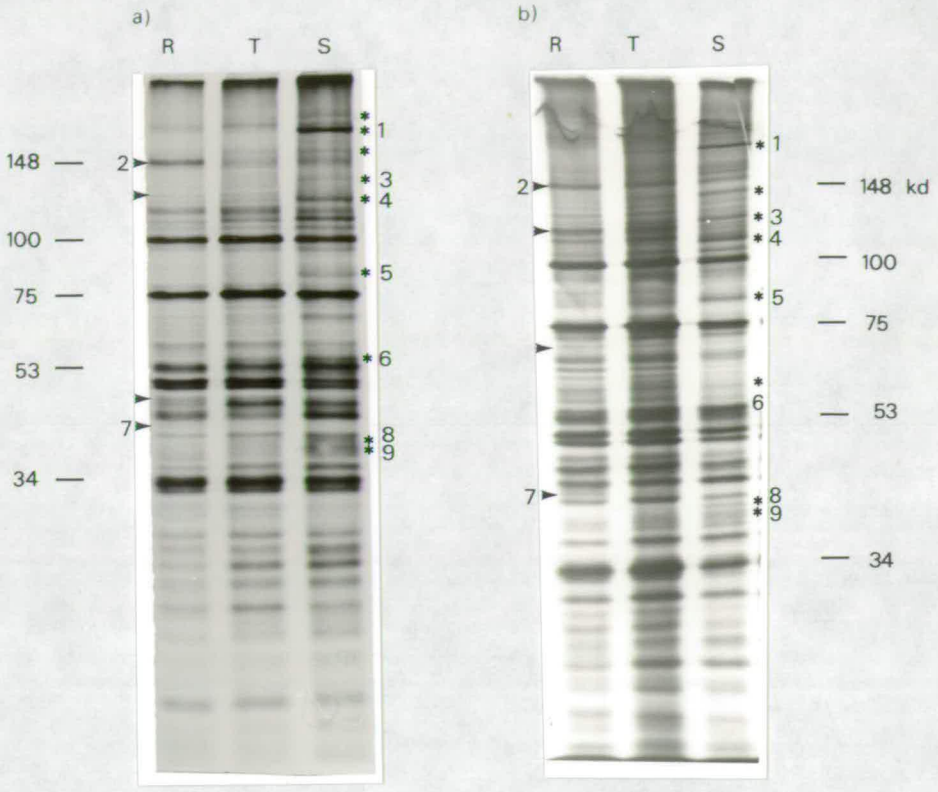
6.3 Electrophoretic Analysis of Stage-specific Protein Synthesis

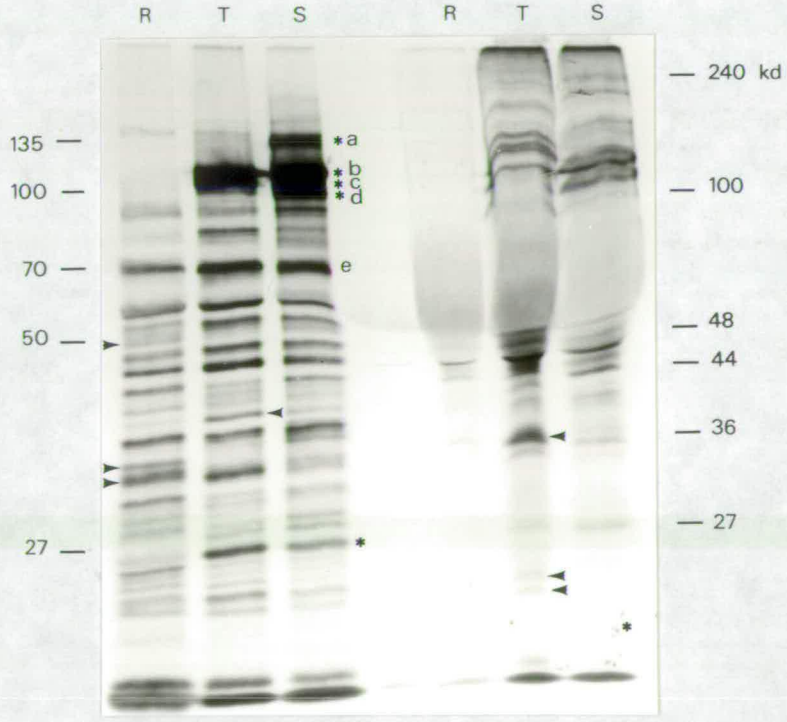
a) One-dimensional gel electrophoresis

Parasite extracts from synchronous cultures of the identical clones 32 and 94 were electrophoresed on one-dimensional SDS-polyacrylamide gels (see section 2.4 for full experimental details). Equal cpm of each labelled sample were compared and autoradiographs of two labelling experiments are shown in fig. 6.4. More than 50 polypeptides were resolved on these gels. The majority (about 35) were seen in extracts of all three parasite stages and appeared to be synthesised throughout the cell cycle. These included most of the strongly-labelled parasite proteins. The remaining proteins showed varying degrees of stage-specific labelling and are indicated in Fig. 6.4. Analysis of all possible stage-specific differences seen on these gels identified twelve polypeptides which were synthesised only by schizonts or by trophozoites and schizonts; one synthesised predominantly by trophozoites; and six by rings or by rings and trophozoites. However, only eight of these stage-specific differences were common to both labelling experiments. These included six polypeptides (bands 1, 3, 4, 5, 8 and 9 on fig. 6.4) found predominently in extracts of mature parasites (molecular weights of 178, 138, 121, 84, 40 and 39 kd respectively) and two (2 and 7) found mainly in rings (148 and 42 kd). Of these polypeptides, the 178 kd schizont-specific and the 148 kd ring-specific proteins are major parasite components. The differences between labelling experiments,

Fig. 6.4 1D gels of ring (R), trophozoite (T) and schizont (S) labelled parasite pellets from cultures of T9 c94 (a) and c32 (b). * , proteins synthesised by mature parasites; ► , those synthesised by young parasites. Bands 1-9 are discussed in the text.

Fig. 6.5 1D gel of ^{35}S-methionine labelled proteins in saponin supernatants (left hand tracks) and culture medium (right hand tracks) from ring (R), trophozoite (T) and schizont (S) stage cultures of c32. * , proteins synthesised mainly by mature parasites; ◄ , those synthesised mainly by trophs; and, ► , by rings. Bands a-e are referred to in the text.


a)
R
T
S
148
100
75
53
34
2
7
1
3
4
5
6
8
9
b)
R
T
S
2
7
1
3
4
5
6
8
9
148 kd
100
75
53
34


R
T
S
R
T
S
135
100
70
50
27
a
b
c
d
e
240 kd
100
48
44
36
27

e.g. band 6 in fig. 6.4, may have been the result of differences in the exact periods of labelling and conditions used.

Solubilised samples of saponin supernatants (see section 6.2) were also analysed by one-dimensional gel electrophoresis as illustrated in fig. 6.5. Over forty labelled polypeptides were resolved and at least 23 of these were common to all stages of the synchronous culture. Many, if not all, of the resolved polypeptides probably correspond to bands found in extracts of parasite pellets. However, the relative intensities of the labelled bands is strikingly different. In saponin supernatants from synchronised cultures, the dominant labelled bands had molecular weights of 135, 114, 105, 102 and 70 kd (bands a-e, fig. 6.5). The first four of these were all labelled exclusively by mature parasites and are probably equivalent to proteins of similar stage-specificity seen in parasite extracts e.g. bands 3 and 4 in fig. 6.4. Other obvious stage-specific differences include a 38 kd polypeptide labelled most strongly in extracts of trophozoites; bands of 50, 33 and 31 kd synthesised mainly by rings (or rings and trophozoites); and a protein of 27 kd labelled intensely in trophozoite and schizont preparations. (See fig. 6.5, tracks 1-3).

A gel of samples of culture medium from labelled synchronous cultures is also shown in fig. 6.5. The presence of high concentrations of serum proteins in the extracts distorted the banding pattern and made interpretation of the gel difficult. However, only about 25 discrete bands were visualised and the majority of these were

labelled most strongly in the medium from trophozoites and schizonts. 6-10 high molecular weight proteins (100-240 kd) were present in these samples and may include bands a-d already identified in saponin supernatants (fig. 6.5). The lower molecular weight polypeptides included prominent bands of approximately 48, 44, 36 and 27 kd. The 48 and 44 kd proteins appeared in all three stages. The 36 kd, and minor bands of 24 and 23 kd, were more prominent in trophozoite than in schizont preparations. The 27 kd protein is probably the same as the 27 kd band of similar stage-specificity seen in extracts of saponin supernatants.

This 1D gel analysis clearly showed that a number of parasite proteins are synthesised in a stage-dependent manner. A few were synthesised predominantly by rings but most were predominantly or specifically synthesised during the development of trophozoites and/or schizonts (fig. 6.4). As detailed above, some parasite products can be recovered in the medium of labelled cultures, particularly late in development, and many are released following saponin lysis. Although the proteins detected in the saponin supernatants probably correspond to proteins present in free parasite extracts the striking difference in the labelling patterns of these two preparations (see figs. 6.4 and 6.5) indicates that some proteins are preferentially lost during saponin lysis.

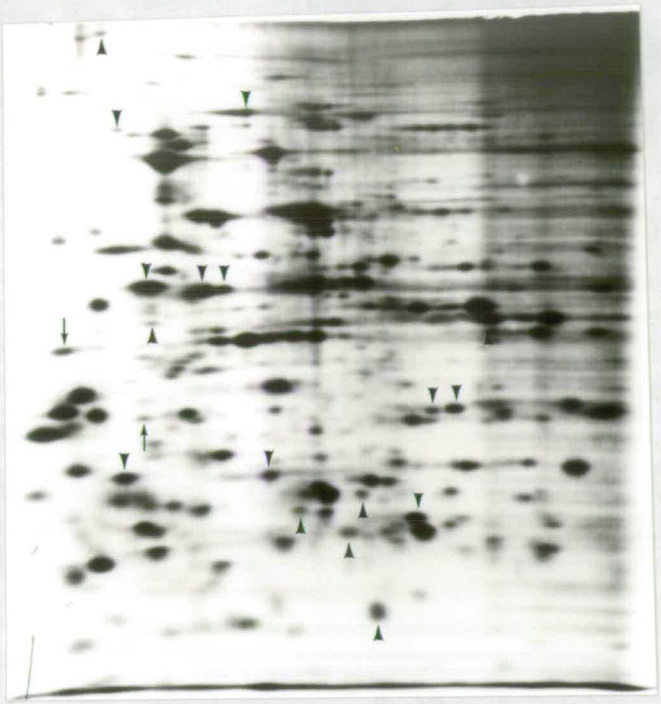
b) Two-dimensional gel electrophoresis

Parasite extracts of labelled, synchronised cultures of T9 clones 32, 94 and 96 were all analysed on two-dimensional gels. Methods of

Fig. 6.6 a)

b)

c)



d)

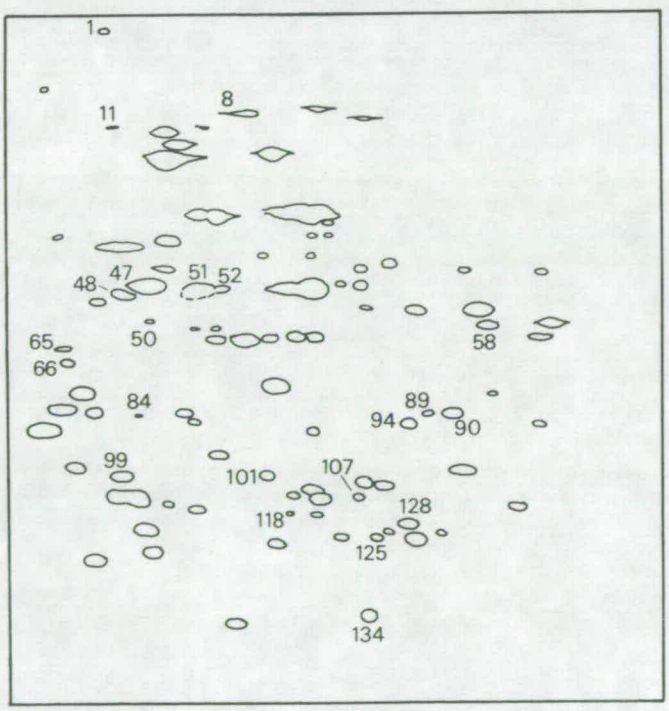
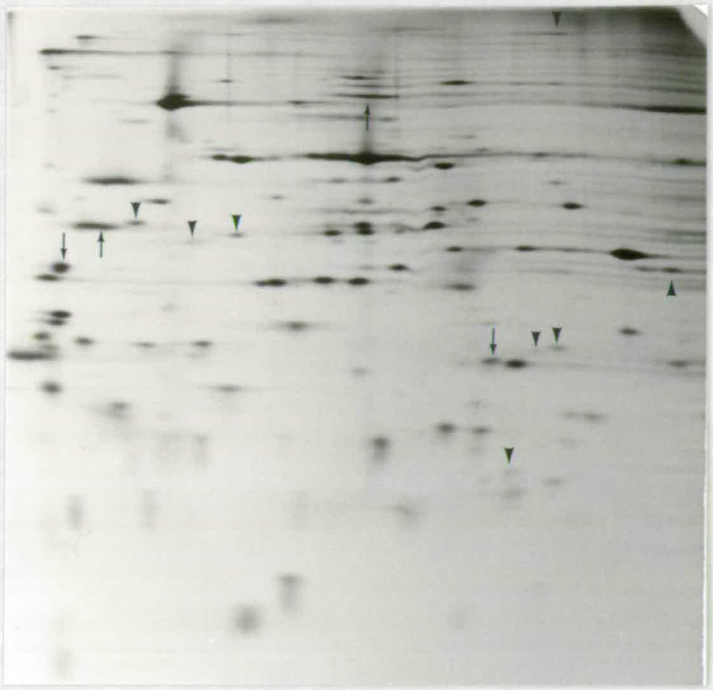


Fig. 6.6 Stage-specific synthesis of proteins in ^{35}S-methionine labelled cultures of T9 c96. 2D gels of saponin released parasites: a) rings, b) trophozoites, c) schizonts. Diagram (d) shows the position and identity of the stage-specific proteins seen in a)-c). ↓, proteins synthesised predominantly by R or R+T. ▾, S or T+S specific proteins (see also Table 6.1).

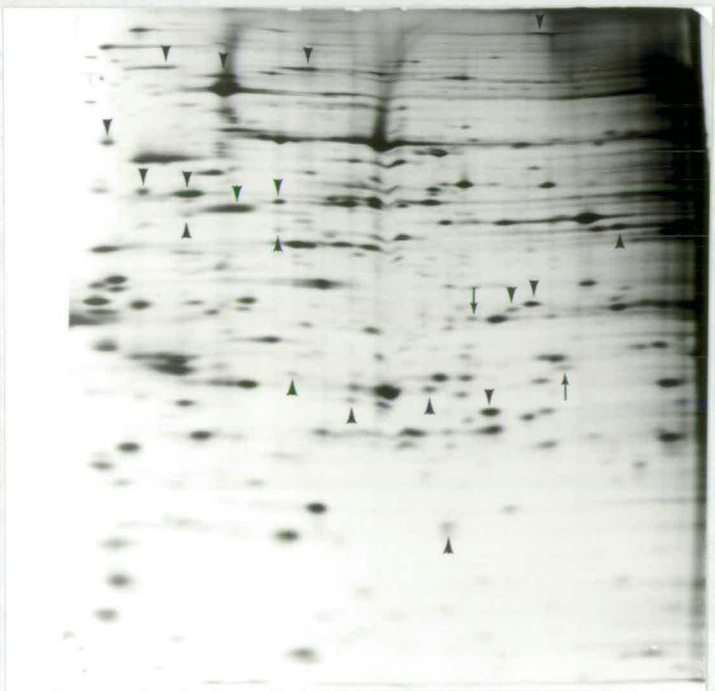
sample preparation, gel electrophoresis and autoradiography were as described in sections 2.3 and 2.4. Initially, analysis of the gels was confined to the set of major, reproducible proteins previously defined for isolate SK17 (see Chapter 3). However, obvious differences in the stage-specific synthesis of seven other proteins were also included in this study.

Autoradiographs of synchronous, labelled cultures of clones 32 and 96 are shown in figs. 6.6 and 6.7. Proteins which appeared to be synthesised to different extents during the three labelling periods are marked on the gels and are identified on the diagrams in figs. 6.6 (d) and 6.7 (d). Analysis of the results of three separate labelling experiments identified as many as 37 proteins which exhibited some degree of stage-specific synthesis. However, only 30 proteins - 28% of those screened - gave the same stage-specific pattern of labelling in more than one experiment and have been considered further. These are listed and characterised in Table 6.1. Some major differences were observed between labelling experiments. Proteins #13, 47, 49, 52, 62 and 63 appeared to be synthesised to the same extent by all stages of the culture of clone 96 whereas they were clearly seen to be synthesised predominantly by mature parasites of both clones 32 and 94. It is likely that this was due to the cultures of clones 32 and 94 being more highly synchronised than clone 96 and due to differences in timing of the three labelling periods (fig. 6.2) rather than being a real strain-specific effect. Hence these six proteins have been classed as probably subject to stage-specific synthesis.

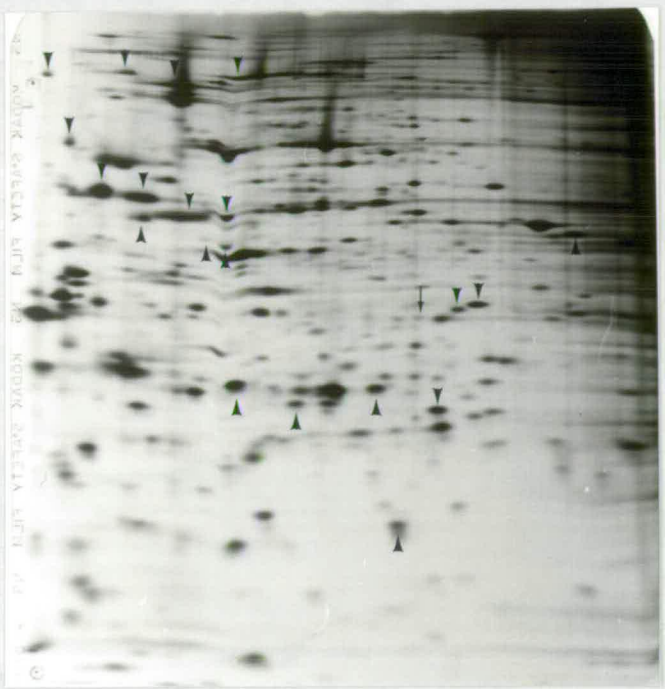
Fig. 6.7 a)



b)



c)



d)

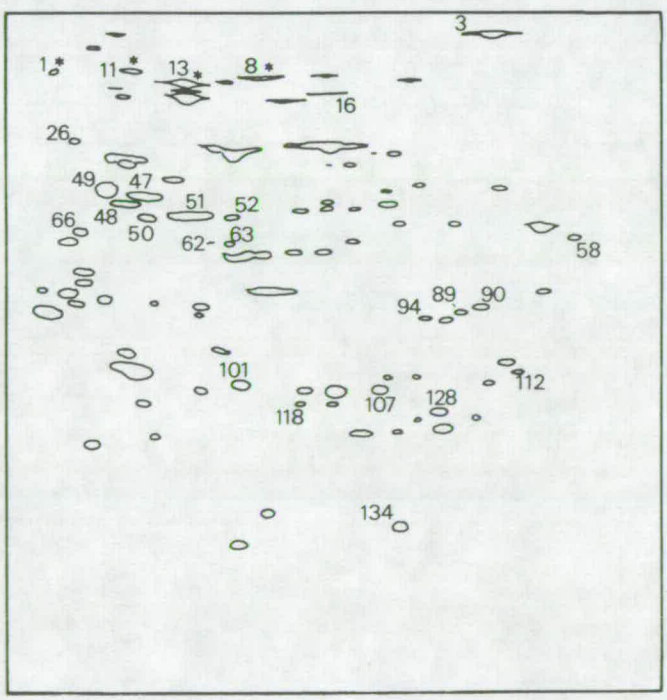


Fig. 6.7 Stage-specific synthesis of proteins in ^{35}S-methionine labelled cultures of T9 c32. 2D gels of saponin released parasites: a) rings, b) trophozoites and c) schizonts. Diagram (d) shows the identity of the stage-specific proteins. ↓ , proteins synthesised predominantly by R or R+T. ▾ , T+S or S-specific proteins. * proteins also present in saponin supernatants (fig. 6.8). See also Table 6.1.

Table 6.1 Parasite proteins subject to stage-specific synthesis

Pattern of stage-specific synthesis			Protein #	Major, reproducible protein	Strain variation(ii)	=band on 1D gels(iii)
R	T	S(i)				
◯	–	–	16	+	+b	
			48			
◯	◯	o/–	66	+	+	
			84	+		
			94	+		
◯	◯	o/–	65			
	◯	o/–	112		+b	
			11		+	
			26		+	
–/o	◯	◯	52a	+		
			99	+		
			128	+		
			1	+	+(B)	
			3	+	+(A)	Band 1
			8	+	+(C)	Band 4?
			13a	+	+(E)	
			47a	+		
			49a	+		
			50	+	+(H)	
			51	+		
–/o	◯	◯	58	+		
			62a	+		
			63a	+		
			89			
			90	+		
			101	+	+(K)	
			107	+		
			118	+		
			125	+		
			134			

(i) The relative intensities of labelling during the development of rings (R), trophozoites (T) and schizonts (S) are indicated by different sized circles (see figs. 6.6-7).

(ii) Variant proteins used for strain-typing (A-M, chapter 4) are marked.

(iii) fig. 6.4

a - these proteins appeared to be stage-invariant in one experiment using T9 c96.

b - these proteins were present in some isolates but absent from others. The other strain-variant proteins showed altered electrophoretic mobilities in different isolates.

Among the proteins listed in Table 6.1 three main types of stage-specific synthesis were seen: i) synthesis exclusively or predominantly by ring stage parasites or by rings and trophozoites, ii) synthesis mainly by trophozoites and iii) synthesis predominantly by mature parasites (trophozoites and/or schizonts). 6 proteins fell into the first category, 1 into the second and 23 into the third. These proteins included both major parasite components (e.g. # 3, 8 and 65) and minor ones (e.g. # 94, 125 and 112). Nine of these stage-specific proteins have previously been observed to vary electrophoretically between isolates (see Table 4.1 and Appendix 2) and include proteins A, B, C, E, H and K used for strain typing.

Samples of saponin-supernatants prepared from synchronous cultures of clone 32 were also analysed by two-dimensional gel electrophoresis (fig. 6.8). The gels were calibrated by direct pH measurements on the first-dimension iso-electric focusing gels and by running molecular weight markers on the second-dimension SDS gels (section 2.7). This allowed the resolved proteins to be provisionally identified. In most cases the identity of the proteins was confirmed by co-electrophoresis of a sample of the saponin supernatant with one of a whole parasite extract. Equivalent auto-radiographic exposures were made of samples from ring, trophozoite and schizont-stage cultures. 30-40 polypeptides were seen on each gel, however only the 17 most intensely labelled ones have been considered in detail. The probable identity of these proteins and their characteristics are given in fig. 6.8 and Table 6.2. Half of

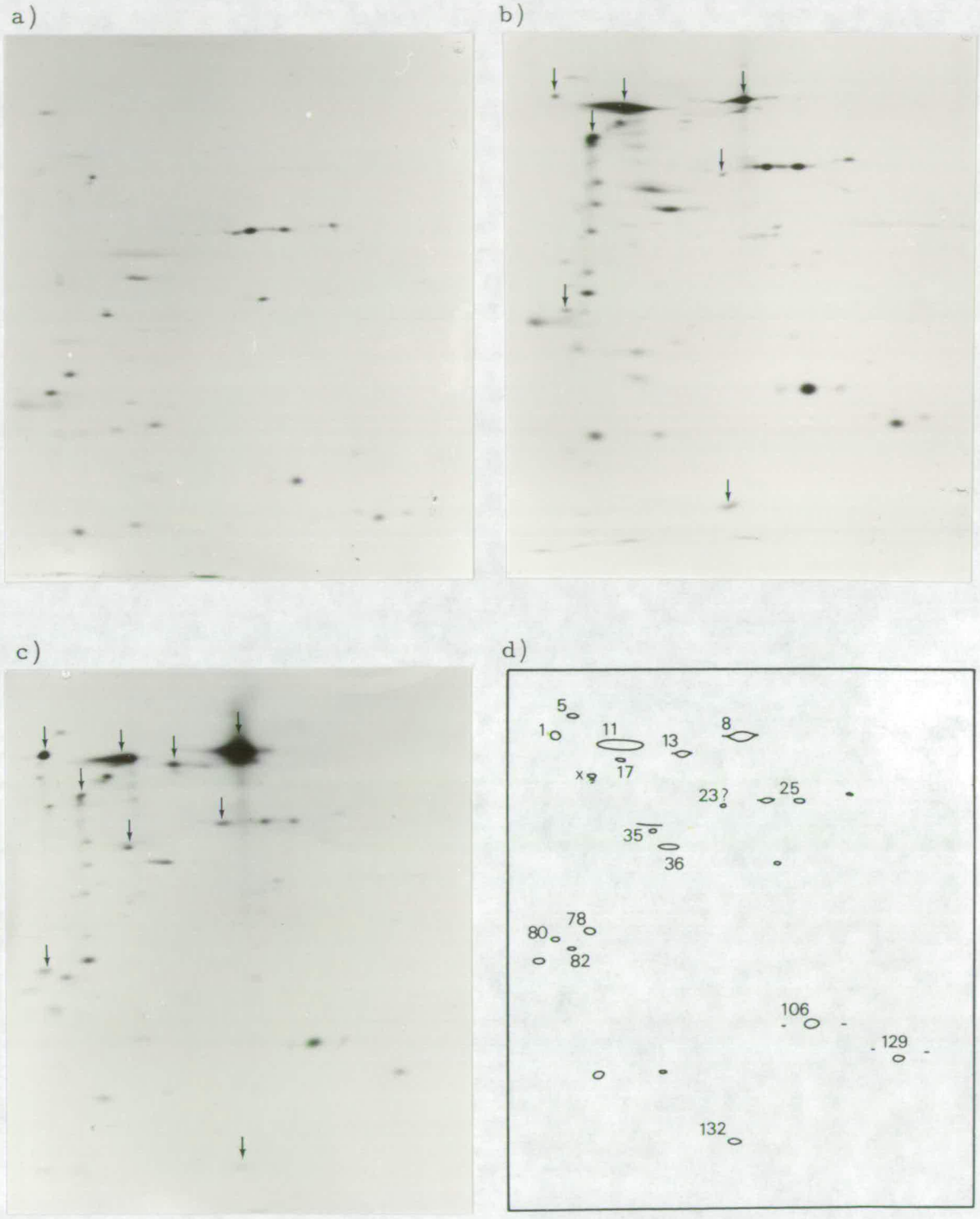


Fig. 6.8 ^{35}S-methionine labelled saponin supernatants from synchronous cultures of T9 c32. 2D gels of a) rings, b) trophozoites and c) schizonts. The identities of these proteins are shown in d). Arrows denote the stage-specific appearance of proteins in this fraction. (See Table 6.2).

Table 6.2 Parasite proteins detected in saponin supernatants

Intensity of labelling in supernatants R T S[i]	Protein #	Major protein in saponin supernatant	Stage-specific synthesis[ii]	Strain variation	≡ band on 1D gels[iii]
○ ○ ○	5		I		
	17		I?		
	25	+	I		
	36	+	I	+(G)	
	78		I		
	82		I		
	106	+	I		
	129		I	+(L)	
-/o ○ ○	11	+	T,S	+	band c?
	x	+	?		
	132		I		
- -/o ○	1	+	S	+(B)	
	8	+	S	+(C)	band b?
	13		S	+(E)	band d?
	23?		I		
	35		S		
	80		S		

(i) see fig. 6.8.
(ii) T or S: synthesised by trophozoites or schizonts. I: stage-invariant, synthesised throughout cycle.
(iii) see fig. 6.5.

these proteins were found in samples of all three parasite stages (# 5, 17, 25, 36, 78, 82, 106 and 129) while the other half were present mainly in trophozoite and/or schizont extracts (#1, 8, 11, 13, 23, 35 and 80). The majority of the proteins corresponded to proteins of similar stage-specificity seen on gels of parasite extracts of the same synchronous culture. These have been indicated in fig. 6.7 (d). The exceptions were two proteins strongly labelled in the schizont saponin supernatants: one appeared to migrate to the same position as the stage-invariant protein #23 and the other ($\varkappa$, fig. 6.8) could not be positively identified in parasite extracts. Proteins # 1, 8 and 11 are the most intensely labelled proteins in this fraction.

Samples of medium from cultures of rings, trophozoites and schizonts were also analysed by two-dimensional gel electrophoresis as described for the saponin supernatants. The results are shown in fig. 6.9. Although equivalent exposures were made for each autoradiograph very few proteins were detected in the medium from ring-stage cultures, whereas 25-30 proteins were seen on gels of the trophozoite and schizont samples. The probable identities of these proteins are shown in fig. 6.9. It can be seen from a comparison of these gels with those of solubilised parasite proteins presented in fig. 6.7 that most of the proteins detected in the medium correspond to intensely labelled proteins seen in free parasites. These include stage-invariant proteins, e.g. # 23, 25, 36, 70, 106 and 129, and proteins specific to mature parasites, e.g. 8, 13,

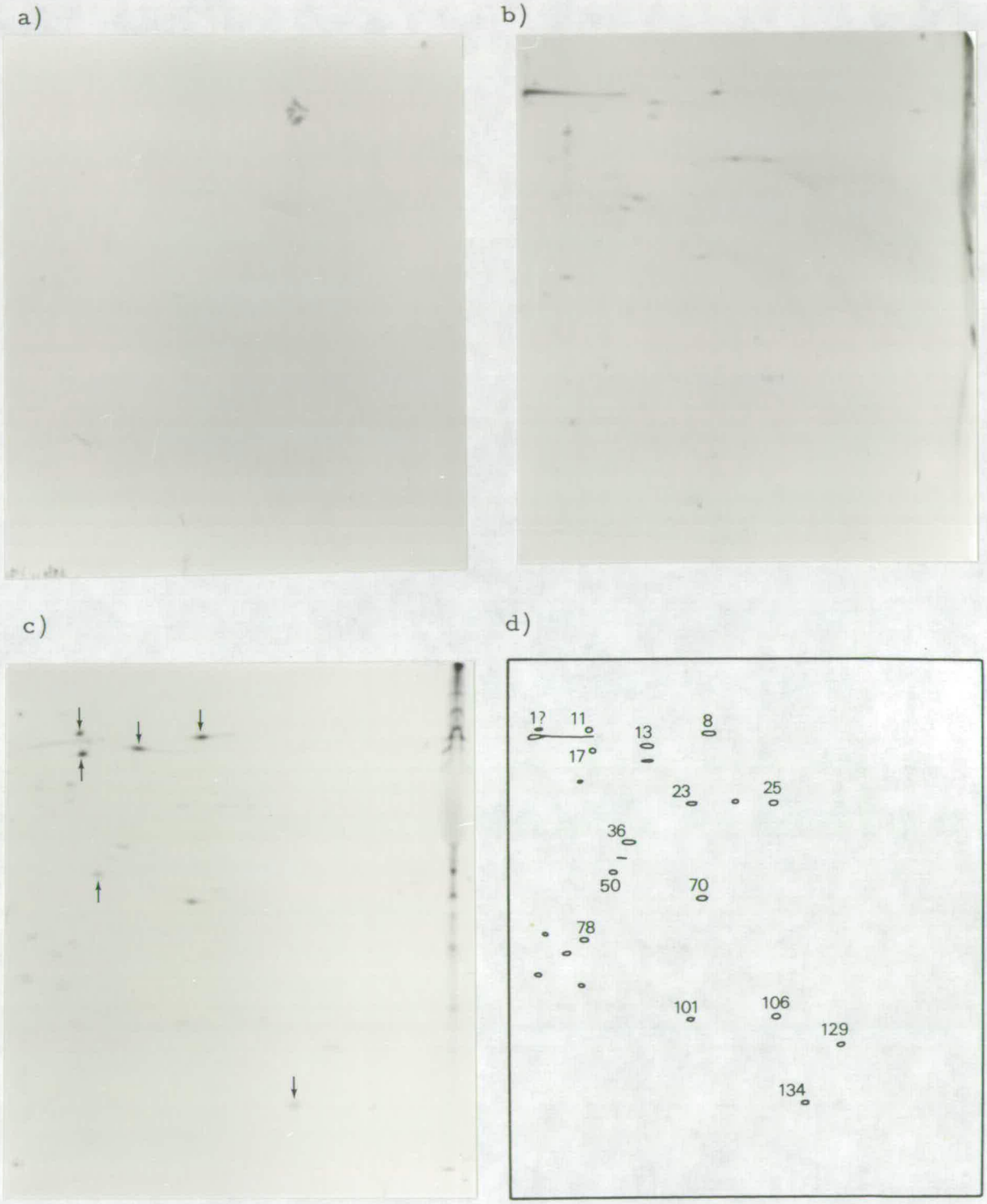


Fig. 6.9 ^{35}S-methionine labelled medium from synchronous cultures of T9 c32. 2D gels of a) rings, b) trophozoites and c) schizonts. The identities of these proteins are shown in d). Proteins arrowed are more intensely labelled in medium from schizont-stage cultures than in that from trophozoite cultures. (See Table 6.3).

Table 6.3 Parasite proteins detected in culture medium

Intensity of labelling in medium of R T S[i]	Protein #	Strongly labelled in: parasite pellet[ii]	Strongly labelled in: saponin supernatant[iii]	Stage-specific synthesis[iv]	Strain variation
-/o ◯ ◯	1?	+	+	S	+(B)
	36	+	+	I	+(G)
	70	+		I	
- -/o ◯	8	+	+	S	+(C)
	11	+	+	T,S	+
	13	+	+	S	+(E)
	17		+	I	
	50	(+)		S	+(H)
	134	+		S	

(i) see fig. 6.9.
(ii) shown in figs. 6.6 & 6.7.
(iii) see fig. 6.8.
(iv) see note (ii), Table 6.2.

101 and 134. However, the relative intensity of labelling of proteins resolved on 2D gels of solubilised culture medium is not identical to that of free parasites. Some proteins are relatively more intensely labelled in samples of culture medium and may, therefore, be selectively accumulating in this fraction. A set of nine such proteins are detailed in Table 6.3. These include proteins #50, 70 and 134 in addition to six others, #1, 8, 11, 13, 17 and 36, which were also abundant in the saponin supernatants. Six of these proteins (#1, 8, 11, 13, 50 and 134) were synthesised predominantly by mature parasites and three (#17, 36 and 70) showed no stage-specificity.

The results of this 2D gel study show the same general trends as revealed by 1D gel electrophoresis but have allowed a much more detailed analysis to be made of the stage-specific synthesis of parasite proteins. Over 28% of the proteins identified in parasite extracts showed some degree of stage-specificity with a few proteins being synthesised mainly early in development but with the majority being synthesised predominantly by trophozoites and schizonts (Table 6.1). Analysis of medium and saponin supernatant from labelled cultures has identified nine proteins which may be preferentially accumulating in the medium of trophozoite- and schizont-stage cultures (Table 6.3) and a set of 17 proteins which seem to be selectively lost during the preparation of free parasites by saponin lysis (Table 6.2). Both these sets of proteins include a high proportion of schizont-specific and strain-variant proteins. These results and their relevance to the 2D gel studies described in previous chapters are considered in more detail below.

6.4 Discussion

Stage-specific protein synthesis

It is well established that the incorporation of labelled amino acids into parasite protein is stage-dependent. Cultures of ring forms synthesise relatively little protein whereas mature parasites produce much more (section 6.3; Perrin et al, 1981a; Brown et al, 1982a; Boyle et al, 1983b) with the peak rate of synthesis occurring when parasites are late trophozoites or early schizonts (Rodriguez Da Silva et al, 1983; Deans et al, 1983b). One-dimensional gel electrophoresis has been used extensively to analyse the spectrum of proteins synthesised during development (Kilejian, 1980a; Perrin and Dayal, 1982; Brown et al, 1982a; Myler et al, 1982 and 1983; Allred and Sherman, 1983; Boyle et al, 1983b; Deans et al, 1983b). It is difficult to make detailed comparisons of these studies since the use of different parasite isolates, different labelling protocols and different methods of harvesting and solubilising parasites all affect the final pattern of labelled proteins seen on the gels. However, most workers have reported the same general findings: cultures of rings and trophozoites synthesise very similar sets of proteins; and the onset of schizogony coincides with the appearance of several new, predominantly high molecular weight, proteins. These conclusions can also be drawn from the one-dimensional gels illustrated in fig. 6.4. Some proteins however are synthesised specifically or predominantly by young parasite stages (section 6.3; Myler et al, 1982).

The more detailed analyses of Boyle et al (1983b) and Deans et al (1983b) show that even during the growth of rings or trophozoites several different stages of development can be characterised by minor changes in the proteins being synthesised.

The results of the two-dimensional analysis presented in section 6.3 revealed a similar pattern of stage-specific synthesis to that shown by one-dimensional gel electrophoresis. Many more stage-specific proteins - including minor parasite components - were detected. As many as 37 possible stage-dependent proteins were identified by two-dimensional gel analysis whereas a maximum of 19 were seen by one-dimensional techniques. Those characterised on two-dimensional gels are detailed in Table 6.1 and some correspond to polypeptides of similar stage-specificity seen on the one-dimensional gels e.g. protein spot #3 in figs. 6.6 and 6.7 and band 1 in fig. 6.4 (see also Table 6.1). However, these two techniques may be examining slightly different sets of parasite proteins. Band 2, for example, in fig. 6.4 appeared to be an intensely labelled protein synthesised predominantly by early parasite stages. However no equivalent protein spot could be identified on two-dimensional gels. The solubility or pI of this protein, and perhaps of others, may have precluded its appearance on the two-dimensional gels analysed.

Two types of stage-dependent synthesis were observed. Firstly, the synthesis of some proteins, e.g. # 16, 49 and 65, was restricted to certain labelling periods and, secondly, other proteins

appeared to be synthesised during all periods but were only strongly labelled during certain periods. In fact the majority of stage-specific proteins characterised in Table 6.1 are of the second type. Allred and Sherman (1983) have also distinguished between true stage-specific proteins which are synthesised discontinuously during erythrocytic development and others whose synthesis is not discontinuous but is modulated in a stage-dependent manner. However, in the experiments described in this chapter it is possible that many of the stage-dependent proteins identified do have a discontinuous pattern of synthesis over the erythrocytic cycle but that this has been masked by the labelling protocol used. Since labelling was carried out over three long and consecutive periods with partially overlapping parasite age-ranges this would be expected to give low levels of labelling in one interval of true stage-specific proteins synthesised predominantly in another.

The majority of stage-dependent proteins were synthesised by mature parasites. Of 30 proteins characterised (Table 6.1) 5 were synthesised exclusively or predominantly by ring stages; 1 by rings and trophozoites; 1 by trophozoites only; 5 by both trophozoites and schizonts; and 18 by schizonts. The same trend was revealed by one-dimensional gel analysis (section 6.3; Deans et al, 1983b; Myler et al, 1983). Thus the appearance of many new proteins correlates with the many metabolic, antigenic and morphological changes which characterise the developing schizont.

A high proportion of the stage-specific proteins identified on

2D gels are also strain-specific. Of the 30 stage-specific proteins analysed 9 have already been observed to vary electrophoretically between isolates (see Tables 4.1, 6.1 and Appendix 2). Of these strain-specific proteins 2(# 16 and 66) were synthesised predominantly by rings, 3 (# 11, 26 and 112) by trophozoites and schizonts and 6 (# 1, 3, 8, 13, 50 and 101) by schizonts. These schizont-specific proteins included proteins A, B, C, E, H and K used for strain typing. Other strain typing proteins (D, F, G, I, J, M and L) were synthesised throughout the cycle. It is of interest that those proteins which had simple types of electrophoretic variation, e.g. the pI shifts of F, I, J, L and M (section 4.3), were synthesised at all stages of development whereas those which had more complex patterns, e.g. A, B, C, H and K, were mostly synthesised late in development. Two stage-specific proteins noted above - # 16 and 112 - had been classed as being present in some isolates but absent in others (Appendix 2). # 16 was synthesised predominantly by rings and # 112 by trophozoites. The stage-spectrum of parasites in asynchronous cultures could thus affect the intensity of labelling of these proteins and this raises the question of whether the presence/absence of these proteins is a good strain typing character. Both proteins were present in extracts of clones 32 and 94 but neither was detected at any stage of development in synchronous clone 96 cultures analysed here. Therefore, at least in this case, the absence of these proteins is a reproducible strain difference.

Protein loss during labelling and saponin lysis

Another question which was investigated with the aid of synchronous cultures was the loss of parasite proteins during the process of labelling cultures and harvesting the parasites. Relatively large amounts of TCA-precipitable labelled material are lost into the culture medium and during saponin lysis both of asynchronous (Table 3.3) and synchronous cultures (fig. 6.3). Therefore, it was of interest to compare the stage-specificity and distribution of proteins in the culture medium and saponin supernatant with those of free parasites derived from ring-, trophozoite- and schizont-stage cultures.

Both 1D and 2D gel analysis revealed striking differences between extracts of free parasites (figs. 6.4 and 6.7) and saponin supernatants (figs. 6.5 and 6.8). The latter consisted of relatively large amounts of a restricted set of the proteins present in free parasites. In particular, proteins #1, 8 and 11 were very strongly labelled in the saponin supernatants and proteins # 13, 36, 106 and 129 were also well represented. The characteristic labelling pattern seen on gels of this fraction suggests that most of the proteins detailed in Table 6.2 are selectively lost during saponin lysis and are not simply the result of a general loss of parasite protein. A high proportion of stage-specific and strain-variant proteins were among those identified in saponin supernatants. Of the 17 proteins characterised in Table 6.2, 6 were synthesised predominantly by trophozoites and/or schizonts and 6 varied

electrophoretically between isolates. In addition, several of the proteins in this fraction (e.g. # 1, 8, 11 and 13) correspond to proteins whose relative intensity of labelling was markedly diminished by labelling parasites in culture rather than as free parasite suspensions (Chapter 3, figs. 3.1 and 3.2). The evidence presented here confirms that at least some of the differences in the patterns of labelling produced by the two labelling procedures are due to the selective loss of proteins during saponin lysis. The possible reasons for this have been discussed in Chapter 3 and it is conceivable that the loss of specific proteins is correlated with their sub-cellular location e.g. they may be associated with the erythrocytic cytoplasm or membrane. If this is the case, then the prevalence of schizont-specific, strain-variant proteins among them may be of immunological significance. This possibility is discussed more fully in Chapter 9.

Analysis of the culture medium collected from labelled synchronous cultures gave rather different results. Although relatively large amounts of TCA-precipitable material were recovered in the medium of ring, trophozoite and schizont stage cultures (fig. 6.3) this did not seem to accurately reflect the amount of parasite protein present. Both 1D and 2D gel analysis detected low levels of labelled protein in the medium from trophozoite and schizont stage cultures but very little from ring stage cultures (figs. 6.5 and 6.9). As detailed in section 6.3 several of the proteins identified in the medium were intensely labelled components of free parasite extracts and this indicates that the lysis of parasitised cells during labelling or

harvesting gave rise to a generalised loss of parasite proteins into the culture medium. It is possible that some proteins are also selectively accumulating in the medium since nine proteins, detailed in Table 6.3, appear to be relatively more intensely labelled in samples of medium than in free parasites. However, six of these proteins (#1, 8, 11, 13, 17 and 36) are major components of the saponin supernatants and hence their presence in the culture medium may also be attributed to lysis of infected erythrocytes. This leaves only three proteins (#50, 70 and 134) which may be preferentially released into the culture medium. Of these three proteins two are synthesised predominantly by schizonts and one varies between isolates. Rodriguez Da Silva et al (1983) identified six major components of 200, 180, 140, 82, 60 and 45 kd which were released into the culture medium during merozoite release and reinvasion. The 60 and 45 kd proteins were synthesised throughout the erythrocytic cycle and may be equivalent to the stage-invariant proteins #36 and 70 of similar molecular weight identified here.

In order to put the distribution of parasite proteins into perspective it is necessary to know the quantitative, as well as the qualitative, division of proteins between these three crude fractions of labelled culture. Knowing the percentage of each preparation applied per gel and the exposure times allowed for each auto-radiograph it is possible to estimate the number of "parasite-days" exposure of each gel illustrated. If the gels of parasite pellets (see fig. 6.7) are arbitrarily set at one parasite-day's exposure

then the gels of culture medium (fig. 6.9) are equivalent to 0.5 - 1.3 parasite-days and those of saponin-supernatants (fig. 6.8) are 0.2 - 0.6 parasite-days. On this basis, the relative intensities of the proteins seen on the autoradiographs presented in figs. 6.7 - 6.9 suggest that a large amount of labelled protein is released into the supernatant during saponin lysis and that the major proteins found in the saponin-supernatant - notably #1, 8 and 11 - must represent a large proportion of the parasite's pool of these proteins. Furthermore, this analysis shows that the actual amount of protein found in the medium of these cultures is fairly insignificant compared with that released during saponin lysis and that present in the free parasites. This low level of labelled protein in the culture medium is in agreement with other published work. Wilson and Bartholomew (1975) and Rodriguez Da Silva et al (1983) have shown that the appearance of parasite protein in the culture medium coincides with the time of schizont rupture and merozoite release and that very little is lost earlier in development. Thus, as only a small amount of reinvasion had occurred in the schizont-stage culture analysed here, only low levels of released protein were to be expected.

The results presented in this chapter demonstrate the potential value of applying two-dimensional gel electrophoresis to the analysis of the stage-specific synthesis of proteins. The experiments described have enabled the major parasite proteins to be characterised in terms of their approximate period of synthesis. Much more

information concerning the fine details of stage-specific synthesis could be obtained by using several short labelling periods rather than the three long periods used here to cover the asexual cycle (see Boyle et al, 1983b; Deans et al, 1983b). Also it is important to realise that the stage-specific synthesis of proteins is only one factor determining the overall protein composition of the parasite. Once synthesised some proteins are conserved through subsequent development and may be recovered from parasites in the following erythrocytic cycle (Falanga et al, 1982; Boyle et al, 1983a; Deans et al, 1983a; Myler et al, 1983); certain proteins are modified or processed later in the cycle (Holder and Freeman, 1981, 1982; Deans et al, 1983a; Myler et al, 1983); other proteins are subject to stage-specific loss or degradation (Falanga et al, 1982; Boyle et al, 1983a; Myler et al, 1983). Thus the characteristic profile of each stage of the parasite will be determined by the combined effects of new protein synthesis and the processing, degradation and loss of existing proteins. Although complex, all these processes determining the stage specificity of parasite proteins could profitably be investigated further by two-dimensional gel analysis.

THE CHARACTERISATION OF GLYCOSYLATED PARASITE PROTEINS

7.1 Introduction

The identification of parasite glycoproteins is of considerable interest in malaria research as they are often membrane-associated and as such are potentially important antigens. It is well established that the surface of the merozoite is a target for protective immune responses to human, simian and rodent malarias (Miller et al, 1975; Chulay et al, 1981; Epstein et al, 1981; Holder and Freeman, 1981). Glycosylated proteins are likely to be components of the merozoite surface coat (Bannister et al, 1977) and targets for this response (Epstein et al, 1981; Boyle et al, 1982). There is also evidence that parasite glycoproteins may be associated with the membrane of parasitised erythrocytes (Schmidt-Ullrich et al, 1980; Howard et al, 1981). In some cases these glycoproteins are recognised by immune sera (Schmidt-Ullrich et al, 1981). In addition to being of interest in terms of the immune response, glycoproteins provide a further means of biochemically characterising the P. falciparum proteins identified by two-dimensional gel electrophoresis and correlations between glycosylated and strain-variant proteins may shed some light upon the molecular basis of the observed electrophoretic variation.

Several methods of detecting glycoproteins have been applied to malaria parasites and infected erythrocytes. These include

surface labelling techniques (e.g. Howard et al, 1981), metabolic labelling (e.g. Kilejian, 1980a; Schmidt-Ullrich et al, 1980) and lectin binding studies (Seed and Kreier, 1976; Kilejian and Olson, 1979). In order to restrict analysis to those glycoproteins synthesised by the parasite itself, the procedure adopted here was to metabolically label cultures of P. falciparum with ^{3}H-glucosamine. Previous studies had demonstrated that this monosaccharide was incorporated, mainly by mature parasites, into a small set of polypeptides as identified by one-dimensional gel electrophoresis (Kilejian, 1980a; Udeinya and Van Dyke, 1981; Perkins, 1982).

The aims of the work reported here were firstly to analyse glucosamine-labelled proteins by one- and two-dimensional gel electrophoresis, secondly to study their stage-specific synthesis, and thirdly to identify any glycosylated proteins among the parasite proteins previously characterised (Chapters 3, 4 and 6).

7.2 Metabolic Labelling with ^{3}H-Glucosamine

The experiments described here were carried out on two identical cloned lines - c 32 and c 94 - derived from isolate T9 which have been extensively characterised in previous chapters. Asynchronous cultures were labelled by incubating with 50 μCi/ml of D - [6 - ^{3}H] - glucosamine hydrochloride (22.6 Ci/mmol, Amersham International) in complete RPMI medium for 20-24 hours as detailed in section 2.2. Giemsa stained blood smears were made before and after labelling to monitor the growth and

Table 7.1 ^{3}H-glucosamine labelling of synchronous T9c94(i)

Stage labelled (# hours labelled)	% parasite stages before and after labelling(ii)					Specific activity of labelling (cpm/ 10^8 parasites/ 10 hours)
	Young rings	Late rings	Early trophs	Late trophs	Schizonts	
Rings(15)	97	3				0.54×10^5
	5	88	5	2		
Trophozoites(6)		15.5	84		0.5	1.2×10^5
		4	28	63	5	
Schizonts(11)		0.5	19	70.5	10	2.0×10^5
	31		1.5	10	57.5	

(i) Cultures were incubated with 67 µCi/ml ^{3}H-glucosamine for the intervals indicated. A 1D gel of the solubilised parasites is shown in fig. 7.1.

(ii) Young ring forms were differentiated from late rings by the thicker cytoplasmic 'rings' in older parasites and the trophozoite stages were subdivided on the basis of size.

morphology of the parasites. Synchronous cultures were produced by repeated sorbitol treatments (section 2.1) and were labelled over periods equivalent to those previously used to analyse stage-specific protein synthesis (section 6.2). Three stages of parasite growth were labelled corresponding to the development of rings (approximately 5-20 hours after reinvasion), trophozoites (26-32 hours) and schizonts (32-43). The developmental age difference of parasites in the synchronous cultures was approximately 8 hours. The stage profile of parasites during each labelling period is given in Table 7.1. Parasites were harvested by saponin lysis (section 2.1) and solubilised for one- and two-dimensional gel electrophoresis as detailed in section 2.3. Samples were TCA-precipitated and processed for scintillation counting (section 2.3).

7.3 One-dimensional Gel Analysis

A representative 1D SDS gel of a ^{3}H-glucosamine-labelled extract of an asynchronous culture is illustrated in fig. 7.1, track 1. Five intensely labelled bands can be seen (bands b, d-g) with estimated molecular weights of 165-190, 45-55, 42-49, 34-41 and 29-35 kd. Several weakly labelled bands were also seen on some gels including band c (of slightly lower molecular weight than band b) and a set of 2-3 bands with molecular weights of 25-33 kd (arrowed on track 1).

The results of labelling successive stages of a synchronous culture are shown in fig. 7.1 (tracks 2-4). Approximately equal

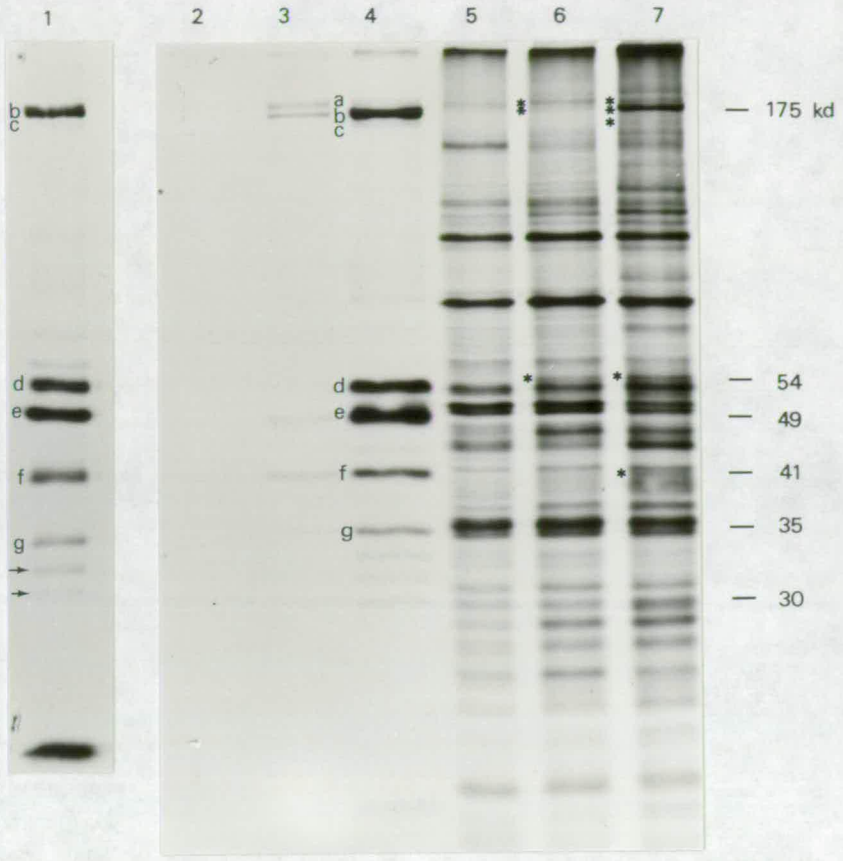


Fig. 7.1 1D SDS gel showing ^{3}H-glucosamine labelled extracts of asynchronous (track 1), ring stage (2), trophozoite stage (3) and schizont stage (4) cultures and ^{35}S-methionine labelled proteins of rings (track 5), trophozoites (6) and schizonts (7) from the same synchronous cultures. Bands a-g and those arrowed are glycosylated proteins referred to in the text. * identifies methionine-labelled proteins which may correspond to glycosylated bands in tracks 1-4.

numbers of parasites were analysed from each of the three labelling periods. No labelled bands were detected in the extract of labelled rings (track 2). Some proteins were glycosylated during the development of trophozoites and early schizonts (bands a, b, e-g, track 3). However it was only during the final labelling period, corresponding to the development of schizonts, that all the intensely-labelled bands (b, d-g) and several minor ones were detected (track 4). In general, the intensity of labelling was lower in the bands appearing in extracts of trophozoites than in those of schizonts. However, one minor band (a, fig. 7.1) was labelled more intensely by trophozoites than by schizonts suggesting that its glycosylation occurred over an earlier and more limited period than that of the other glycoproteins analysed. The banding pattern seen in the extracts of asynchronous cultures was basically the same as that of labelled schizonts (tracks 1 and 4, fig. 7.1) except that some minor components were only apparent in the latter.

Samples of ^{35}S-methionine-labelled extracts of the same synchronous culture were analysed in parallel with the ^{3}H-glucosamine labelled parasites (tracks 5 → 7, fig. 7.1). Comparison of schizonts labelled with these precursors (tracks 4 and 7) indicated that the glycosylated bands b and, possibly, d are major parasite proteins as identified by ^{35}S-methionine incorporation. Four glycosylated bands (a, b, c and f) can be correlated with ^{35}S-methionine-labelled proteins of similar stage-specificity. For example, a protein of the same mobility as band b is strongly labelled in samples of

schizonts, only faintly labelled in trophozoite extracts and is apparently missing from labelled rings. This suggests that these proteins may be glycosylated at approximately the same time as they are synthesised. However, glycosylated band d which was detected only in schizonts was of the same mobility as a ^{35}S-methionine-labelled protein which was strongly labelled in extracts of both trophozoites and schizonts. This raises the possibility that glycosylation may occur several hours after protein synthesis. Although, in view of the limited resolving power of the one-dimensional gel system, it is also possible that the glycoprotein is comigrating with a more abundant, unrelated protein.

7.4 Two-dimensional Gel Analysis

Samples of glucosamine-labelled asynchronous and schizont-stage cultures were prepared and analysed by 2D gel electrophoresis as detailed in sections 2.3 and 2.4. As illustrated in figs. 7.2 - 7.4, five strongly labelled glycosylated proteins were resolved on these gels and up to twenty minor components could also be detected. The labelled extracts of both asynchronous cultures (figs. 7.2 and 7.4) and of schizonts (fig. 7.3) were very similar except that, as indicated in fig. 7.3, some minor components were more intensely labelled in the latter. Nine major parasite glycoproteins, designated gp1 - gp9 in fig. 7.4, have been characterised (Table 7.2). On the basis of their molecular weight and relative intensity of labelling it is likely that gp 1, 4, 5, 7 and 8 correspond to bands b, d, e, f and g identified on 1D gels (fig. 7.1).

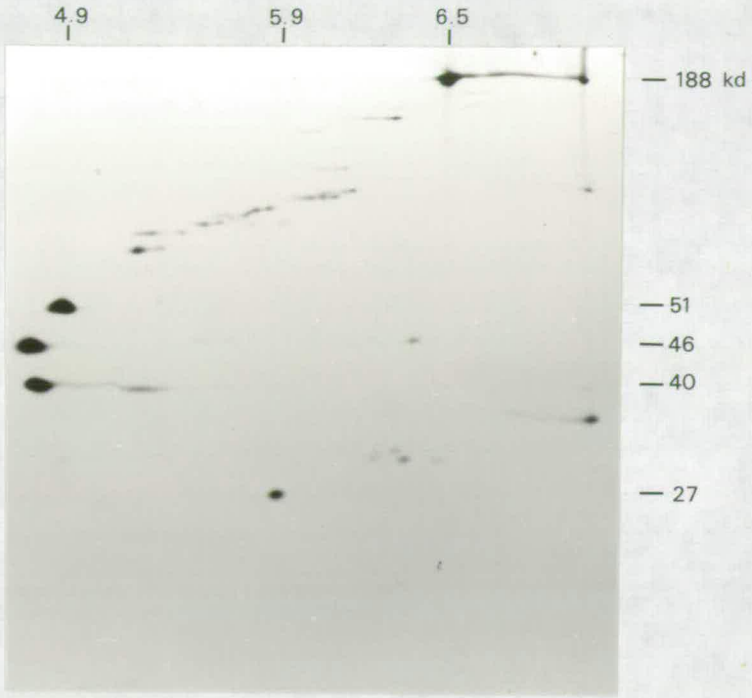


Fig. 7.2 2D gel of a ^{3}H-glucosamine labelled asynchronous culture of T9 c32.

Fig. 7.3 ^{3}H-glucosamine labelled proteins synthesised by schizonts of T9 c32. Those arrowed are labelled more intensely in extracts of schizonts than in extracts of asynchronous cultures.

Fig. 7.4 ^{3}H-glucosamine labelled proteins of an asynchronous T9 c94 culture. The major glycosylated components are numbered gp 1-gp 9.

Fig. 7.5 Co-electrophoresis of ^{3}H-glucosamine and ^{35}S-methionine labelled extracts of T9 c94. The position and identity of several glycosylated components are indicated.

After characterising the glycosylated components in terms of their MW and pI's it was possible to provisionally identify a number of them as amino acid-labelled proteins. Ideally, definitive identification requires double-label analysis of ^{14}C-glucosamine and ^{3}H-amino acid-labelled extracts. However, in this study co-electrophoresis of a ^{3}H-glucosamine-labelled sample with a ^{35}S-methionine-labelled extract (fig. 7.5) was used to try to confirm the identities of these parasite glycoproteins. As indicated in fig. 7.5 at least 13 of the glycosylated components resolved on 2D gels appeared to correspond to ^{35}S-methionine-labelled proteins. Five of the glycosylated proteins detailed in Table 7.2, gp 1, 2, 3, 6 and 7, have been positively identified as proteins #3, 2, 6, 61 and 74 respectively. A further three, gp 4, 5 and 9, have been tentatively identified as proteins #49, 64 and 104, although further work is required to confirm their identities. Gp 8 also corresponded to an amino acid labelled protein (fig. 7.5) but, as it has a very basic pI and did not always focus on the first dimension gels, little else is known about this parasite glycoprotein.

Two of the heavily glycosylated proteins (gp 1 and 4) were also strongly labelled, and therefore abundant, components of amino acid-labelled extracts of asynchronous cultures (proteins #3 and 49). These two proteins and the less strongly glycosylated protein #2 were previously catalogued among the 100 most abundant, reproducible proteins detected in isolate SK17 (fig. 3.10, Appendix 1). The other glycoproteins characterised here all corresponded to

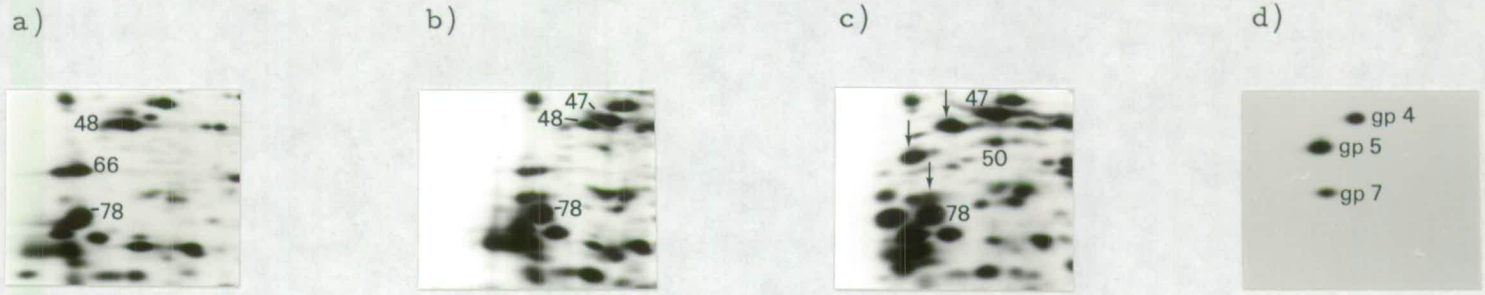


Fig. 7.6 The stage-specific synthesis of ^{35}S-methionine labelled proteins corresponding to gp 4, 5 and 7 in cultures of T9 c94. Sections of 2D gels of ^{35}S-methionine labelled rings (a), trophozoites (b) and schizonts (c) and of ^{3}H-glucosamine labelled asynchronous parasites (d) are shown. The identities of the major proteins are given and schizont-specific proteins thought to be equivalent to gp 4, 5 and 7 are arrowed. (See also fig. 7.5).

minor components of asynchronous cultures and some, including the strongly glycosylated gp 5 and gp 7, could not always be visualised on autoradiographs of amino acid-labelled extracts. These were all, however, more abundant and more easily detected in extracts of amino acid-labelled schizonts. In fact, the majority of the parasite glycoproteins corresponded to proteins (#3, 6, 49, 61, 64 and 74) which were synthesised predominantly by trophozoites and schizonts or by schizonts only (Table 6.1, fig. 7.6) and this is in line with the marked stage-specificity of glycosylation demonstrated in section 7.3. The exception to this trend was protein #2, identified as gp 2, which was synthesised throughout the erythrocytic cycle.

Some of the parasite glycoproteins were identified with proteins which vary electrophoretically between isolates. Others appeared to be invariant. Proteins #2 (gp 2) and #49 (gp 4), for example, did not appear to vary among the isolates screened in Chapter 4, whereas the major glycosylated protein #3 (gp 1) was observed to vary electrophoretically and is the protein A used for strain typing (Tables 4.1, 4.2 and fig. 4.1). Although the other glycoproteins were not included in the analysis of strain variation detailed in Chapter 4, two minor glycosylated proteins - #6 (gp 3) and #61 (gp 6) - were observed to vary in comparisons of the T9 clones and isolate SK17 (detailed in figs. 5.1 - 5.4). The variants of protein #6 differed in both pI and MW whereas those of #61 differed in pI only.

Table 7.2 Characterisation of major parasite glycoproteins

^{3}H-glucosamine labelled proteins:			≡ amino acid labelled protein:				Correspondence with previously characterised glycoproteins (reference[ii])
Identity on 2D gels	band on 1D gels	relative intensity of labelling	#	major, reproducible protein	strain variation	stage of synthesis[i]	
gp 1	b	+++	3(A)	+	+	(T),S	250kd(1); gp185(2); 180-185kd (3); 160kd(4).
gp 2		+	2	+	-	R,T,S	
gp 3		+	6	-	+	T,S	
gp 4	d	+++	49	+	-	(T),S	53kd merozoite gp(3); gp56(2).
gp 5	e	+++	64	-	?	S	44.5kd merozoite gp(3); gp46(2).
gp 6		+	61	-	+	(T),S	
gp 7	f	+++	74	-	?	(T),S	38.5kd merozoite gp(3).
gp 8	g	++	-	-	?	?	gp34(2).
gp 9		++	104?	-	?	?	

(i) during development of rings - R, trophozoites - T, and schizonts - S. (see Chapter 6 and fig. 7.6).
(ii) (1) Kilejian, 1980a; (2) Howard & Reese, 1984; (3) Heidrich et al, 1984; Udeinya & Van Dyke, 1980).

A summary of the relative abundance, stage-specificity and strain-specificity of the main parasite glycoproteins is presented in Table 7.2.

7.5 Discussion

Metabolic labelling with glucosamine or other monosaccharides has been widely used to characterise the glycosylated proteins of *P. falciparum* (Kilejian, 1980a; Udeinya and Van Dyke, 1980, 1981; Perkins, 1982; Heidrich *et al*, 1984; Howard and Reese, 1984) and of other species of *Plasmodium* (Schmidt-Ullrich *et al*, 1980; Newbold *et al*, 1982b; Deans *et al*, 1983a). However, in order to correctly interpret the results of these studies, it is necessary to establish that the labelled precursor is incorporated specifically into parasite rather than host components and into glycoproteins rather than other macromolecules. Udeinya and Van Dyke (1980, 1981) have shown that negligible ^{3}H-glucosamine is incorporated by uninfected erythrocytes. They have also demonstrated that the incorporation of ^{3}H-glucosamine by parasitised cells can be suppressed by tunicamycin, an inhibitor of protein glycosylation, and that even after long labelling periods more than 90% of the incorporated label can be recovered as glycopeptides. Furthermore, the possibility that the labelling pattern might be affected by the metabolism of labelled monosaccharides into amino acids and their subsequent incorporation into parasite proteins has been discounted by Heidrich *et al* (1984). Thus, the available evidence indicates that the main

metabolic fate of glucosamine and other monosaccharides is the synthesis of parasite glycoproteins.

In this chapter the glycoproteins present in parasites prepared by saponin lysis of labelled cultures have been analysed by 1D and 2D gel electrophoresis. In view of the possibility that some proteins may be selectively lost during labelling or harvesting parasites (Chapter 6), samples of medium and saponin supernatants from glucosamine-labelled cultures were also analysed on 1D gels. However, no parasite glycoproteins were detected in either of these fractions (results not shown).

The analysis of glycoproteins synthesised by synchronous cultures (section 7.3) showed that glycosylation occurs predominantly during the development of schizonts and this confirms the findings of Kilejian (1980a). In addition to glycosylation being stage-specific, most of the glycoproteins resolved on 2D gels were identified with proteins which were also synthesised late in the erythrocytic cycle (Table 7.2). Thus, both the synthesis and glycosylation of these proteins was occurring at the same stage of development. However, protein #2 which was thought to correspond to gp 2 was synthesised throughout the cycle and this raises the possibility that glycosylation may occur several hours after synthesis of the protein. More of the fine detail of the stage-specific synthesis and glycosylation of these proteins might be revealed by labelling synchronous cultures with glucosamine or amino acids over several short periods rather than the three long intervals used in this study.

Kilejian (1980a), Udeinya and Van Dyke (1980, 1981), Perkins (1982) and Heidrich et al (1984) have analysed the glucosamine-labelled proteins of P. falciparum by one-dimensional polyacrylamide gel electrophoresis. Direct comparisons of these studies are complicated by the use of different parasite isolates, different methods of harvesting the labelled parasites and different electrophoretic conditions for their analysis. However, all show a similar pattern of labelling with one strongly labelled, high molecular weight glycoprotein and a series of 4-7 glycosylated bands of much lower molecular weight probably equivalent to bands b and d-g in fig. 7.1. Although the labelling patterns were similar, the estimated molecular weights of putatively equivalent glycoproteins were not. For example, the large glycoprotein was estimated to be > 250 kd by Kilejian (1980a), 180-185 kd by Heidrich et al (1984) and 160 kd by Udeinya and Van Dyke (1980). These differences may be the result of the anomalous behaviour of glycoproteins on SDS polyacrylamide gels. Electrophoresis in gels of different % acrylamide can result in the same glycoprotein having different apparent molecular weights (Segrest and Jackson, 1972).

In relation to the cellular location of these parasite glycoproteins, Kilejian (1980a) has demonstrated that in schizont-infected erythrocytes, the high molecular weight glycoprotein (equivalent to band b, fig. 7.1) is accessible to surface labelling reagents. Perkins (1982) claimed that all the parasite glycoproteins were

at the surface of infected cells and that none were associated with merozoites. However, Heidrich et al (1984) have shown conclusively that glycoproteins of 53, 44.5, 38.5 (≡ bands d, e and f, fig. 7.1) and 15 kd are present in isolated merozoites and that the 53, 38.5 and 15 kd components are processing products of the 180-185 kd schizont glycoprotein (≡ band b, fig. 7.1). Comparison of the 1D and 2D gel analyses presented in this chapter indicates that protein #3, resolved on 2D gels, is the major, high molecular weight glycoprotein characterised in the above studies. In addition, gp 4, 5 and 7 (proteins #49, 64 and 74) may be equivalent to the merozoite glycoproteins identified by Heidrich et al (1984) thus implying that gp 4 and 7 (proteins #49 and 74) may be breakdown products of gp 1 (protein #3). Howard and Reese (1984) have also used 2DGE to characterise the glycoproteins of P. falciparum and they identified four major glycosylated proteins of 185, 56, 46 and 34 kd which, on the basis of their MW and pI's, probably correspond to gp 1, 4, 5 and 8 characterised in this thesis.

At the molecular level, another aspect of interest was the possible role of glycosylation in producing electrophoretic variation in proteins of different isolates of P. falciparum. Three proteins - #3, 6 and 61 - which exhibited such variation were found to be glycosylated. Variants of protein #6 differed in pI only whereas those of #3 and 61 differed in both pI and MW. In the case of protein #3 (A used for strain typing) the pI differences were equivalent to single charge changes but the MW differences were very large,

variants A1 and A2 being 8-10 kd larger than A3 and A4. Thus, one possible inference is that the large change in apparent MW is the result of different degrees of glycosylation in different isolates.

The results presented in this chapter have allowed the characterisation of a small set of intensely labelled parasite glycoproteins (Table 7.2). As detailed above, comparisons with other published work have enabled some conclusions to be drawn about the cellular location and molecular relationships of some of the glycosylated proteins resolved on 2D gels. As discussed more fully in Chapter 9, the identification of the strain-variant protein #3 as the major high MW glycoprotein of P. falciparum is of great significance. Similar high MW glycoproteins have been identified in other species of malaria including P. knowlesi and P. chabaudi (Epstein et al, 1981; Newbold et al, 1982b) and these glycoproteins or their merozoite-associated products have been implicated in protective immunity (Epstein et al, 1981; Boyle et al, 1982; Hall et al, 1984a).

THE IDENTIFICATION OF PARASITE ANTIGENS

8.1 Introduction

One obvious extension of the analysis of P. falciparum proteins by two-dimensional gel electrophoresis was to apply the technique to the identification of parasite antigens and thus to determine which parasite proteins can induce a humoral response in humans or experimental animals. Combining immunoprecipitation techniques with the high resolving power of the two-dimensional gel system is an ideal way of analysing the proteins recognised by the immune system. Once such antigens have been identified on two-dimensional gels, then it may be possible to characterise them in terms of their abundance in the parasite, stage of synthesis, variation between strains and possible glycosylation based on the results of the preceeding chapters. There is an increasing use of monoclonal antibodies in the dissection of parasite antigens and in the search for inhibitory or protective antigens. Again two-dimensional gel electrophoresis could very profitably be applied to the characterisation of the target antigens of these monoclonal antibodies.

The main aim of the work presented here was to combine suitable immunoprecipitation procedures with two-dimensional electrophoresis in order to identify parasite proteins specifically recognised by Thai human serum. As a preliminary to this, different immunoprecipitation methods were compared and, in some experiments, serum from rabbits immunised with P. falciparum

was used. In addition, the target antigens of two monoclonal antibodies (2.2 and 7.3 of Hall et al, 1983) were characterised on 2D gels. Both antibodies were known to react with 190 kd polypeptides but whereas 2.2 reacted with all strains and stages of asexual parasites, 7.3 reacted only with mature parasites of certain strains. Therefore, it was of interest to determine whether these antibodies were directed against the same antigen or against different antigens of similar molecular weight.

8.2 Immunoprecipitation of Parasite Proteins

Three well characterised Thai strains, SK17 and two T9 clones (94 and 96), were used as sources of parasite antigens for immunoprecipitation experiments. Cultures were labelled with ^{35}S-methionine as described in section 2.2 and labelled protein was harvested from either infected erythrocytes or from saponin-released parasites by solubilising with Nonidet-P40 (section 2.8).

Anti-*P. falciparum* serum was obtained from three sources. One batch of serum was produced by immunising a rabbit with SK17 parasites prepared by saponin lysis as detailed in section 2.8. A second batch of rabbit serum, prepared against another Thai isolate, K1, was provided by Dr. R. Hall (Molecular Biology Dept., Edinburgh University). Finally, a sample of freeze-dried human serum, known to react positively with *P. falciparum*, was the kind gift of Dr. S. Thaithong. This serum was collected from a donor in northern Thailand who had had malaria several times.

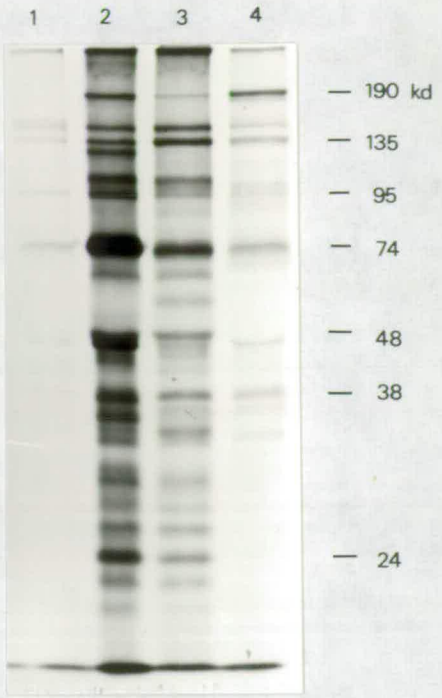
The two procedures used for immunoprecipitating labelled parasite antigens with these sera are described in detail in section 2.8. The first was based on the affinity of staphylococcal Protein A for immunoglobulins (Kessler, 1975 and 1976). The labelled antigen was incubated with the sera to allow immune complexes to form. These were then removed from solution by adsorbing onto Protein A-sepharose. After washing the immuno-adsorbent to remove free antigens, the bound complexes were then eluted, solubilised and analysed by gel electrophoresis. The second method differed in the way that the immune complexes were recovered: rabbit anti-human IgG was used to precipitate the antigen-antibody complexes directly. This method was used only for analysing the parasite proteins recognised by the human serum. The double immunoprecipitates were difficult to solubilise and it was found necessary to modify the normal procedure for two-dimensional gel sample preparation accordingly. This was achieved by first heating the precipitate in 2% SDS and subsequently adding urea and Nonidet-P40 (to give a ratio of SDS:N-P40 of 1:8) as detailed in section 2.3.

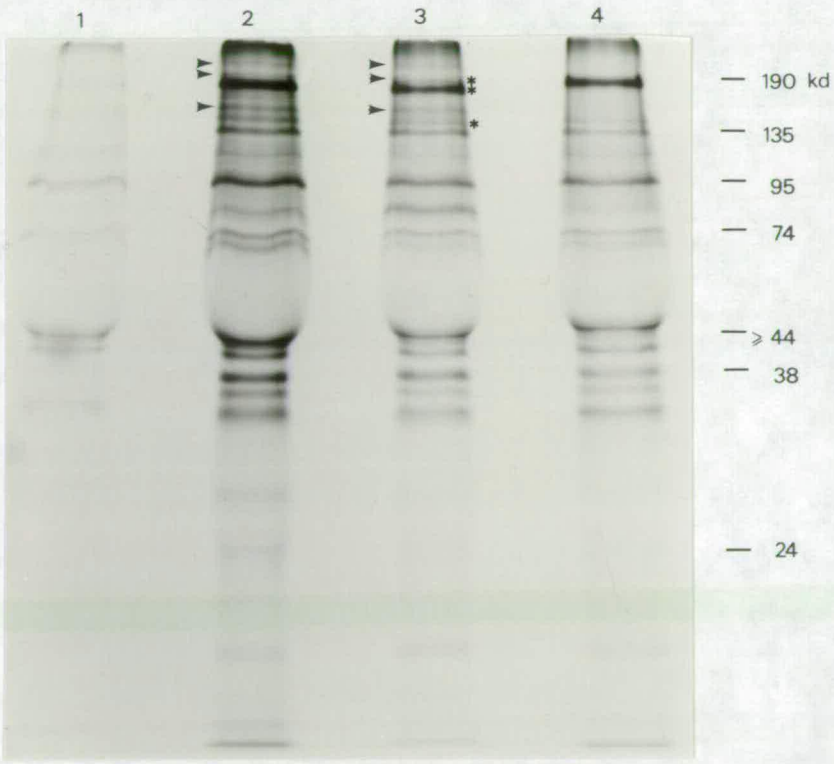
8.3 Electrophoretic Analysis of Immunoprecipitated Proteins

A one-dimensional gel of SK17 proteins recognised by rabbit and human sera using Protein A-sepharose as the immunoadsorbent is shown in fig. 8.1. Normal rabbit serum was used as the control (track 1) and it can be seen that several polypeptides (mostly

Fig. 8.1 Proteins immunoadsorbed onto Protein A-sepharose from ^{35}S-methionine labelled free SK17 parasites by non-immune rabbit serum (track 1), anti-SK17 rabbit serum (track 2), anti-K1 rabbit serum (track 3) and Thai human serum (track 4).

Fig. 8.2 Proteins recognised by Thai human serum by double-immunoprecipitation in ^{35}S-methionine labelled extracts of parasitised erythrocytes of isolate SK17 (track 2), T9 c94 (track 3) and T9 c96 (track 4). Track 1 shows SK17 proteins immunoprecipitated by control European serum. ➤ proteins immunoprecipitated only from the SK17 and c94 extracts. * proteins immunoprecipitated from c94 of lower MW than those from SK17.


1
2
3
4
190 kd
135
95
74
48
38
24


1
2
3
4
190 kd
135
95
74
≥ 44
38
24

corresponding to major parasite components) have been weakly precipitated. The immune rabbit sera, raised against SK17 parasites (track 2) and against K1 (track 3), both immunoprecipitated more than 25 polypeptides. The majority of these were recognised by both sera, although differences in the relative amounts of some proteins bound were apparent. The proteins most strongly recognised by the anti-SK17 serum had molecular weights of approximately 190, 148*, 135*, 126, 107, 95*, 74, 48, 47*, 38*, 35 and 24 kd. Those underlined were major components of the antigen preparations and those marked with an asterisk reacted weakly with the control serum. Most of these labelled proteins were also bound by the anti-K1 serum (compare tracks 2 and 3). However, some striking differences were observed between the two samples. The 190, 126, 47 and 35 kd bands seen by the anti-SK17 serum were only weakly recognised by the anti-K1 serum and, conversely, bands of 57 and 33 kd were more strongly bound by the latter. The proteins bound by the Thai human serum are shown in track 4. About 14 proteins were recognised including several corresponding to bands strongly bound by the rabbit sera, e.g. the 190, 148, 135, 95, 74, 47 and 38 kd proteins. The overall impression given is that the human serum is recognising a similar set of proteins to that seen by the immunised rabbits, although some differences are apparent. For instance, the 190 kd polypeptide is obviously the major component recognised by the human serum whereas it is only one of several proteins strongly bound by the rabbit sera. Also, two polypeptides

of 178 and 103 kd are relatively strongly recognised by the human serum but not by the rabbit sera.

Comparison of tracks 1 and 4 in fig. 8.1, highlights the main problem found with the Protein A-sepharose method, i.e. some of the proteins bound by the control serum produce labelled bands of similar intensity to those recognised by the human serum. This applies particularly to the 148, 95 and 74 kd bands and makes it difficult to determine whether these proteins are specifically recognised by the human serum even though they are clearly highly immunogenic in the rabbit. In order to reduce the level of background precipitation, double immunoprecipitation was undertaken and the result of one experiment is illustrated in fig. 8.2. Tracks 1 and 2 show proteins precipitated from SK17 by the control and the Thai human serum respectively. Comparison with tracks 1 and 4 on fig. 8.1 show that a small number of proteins are still precipitated by the control serum but that the intensity of these bands is reduced relative to those precipitated by the Thai serum. Thus the double immunoprecipitation procedure did appear to reduce the level of non-specific precipitation. However, one disadvantage of this technique is that the large amount of immunoglobulin in the precipitates produces considerable distortion of the 50-70 kd region of the SDS gels. All the proteins precipitated by the Thai serum using the protein A-sepharose method were also detected in the double immunoprecipitates. Some differences were observed between the two samples: for example, the 95 kd antigen was more prominent in the double

immunoprecipitate and two major antigens of 162 and 42 kd were seen only in this sample. These differences may have been due to differences in the antigen preparations themselves or due to different classes and subclasses of immunoglobulin being precipitated by each technique.

The autoradiograph shown in fig. 8.2 also allows comparison of the proteins precipitated by the Thai serum from parasites of SK17 (track 2), T9 clones 94 (track 3) and 96 (track 4). Very similar sets of proteins were precipitated from each strain. Of 21 proteins detected in SK17, 16 were also present in immunoprecipitates of clones 94 and 96. Differences relating to major components of the immunoprecipitates were i) the strongly labelled 190 kd protein seen in the SK17 and clone 96 samples migrated with a molecular weight of 180 kd in the clone 94 sample and ii) the 162 kd protein seen in both SK17 and clone 94 was not detected in the clone 96 sample. These and other differences between minor components of the immunoprecipitates are indicated in fig. 8.2.

Samples from the same experiment were also analysed on two-dimensional gels (fig. 8.3). The identities of the immunoprecipitated proteins were deduced from their molecular weights and positions in creatine phosphokinase charge trains by the methods described in section 2.7. Eleven of the proteins precipitated from extracts of SK17 by the Thai human serum have been provisionally identified as proteins #3, 5, 13, 18, 23, 25, 36, 40, 50, 70 and 101. Those underlined were major components of the immunoprecipitates and #3

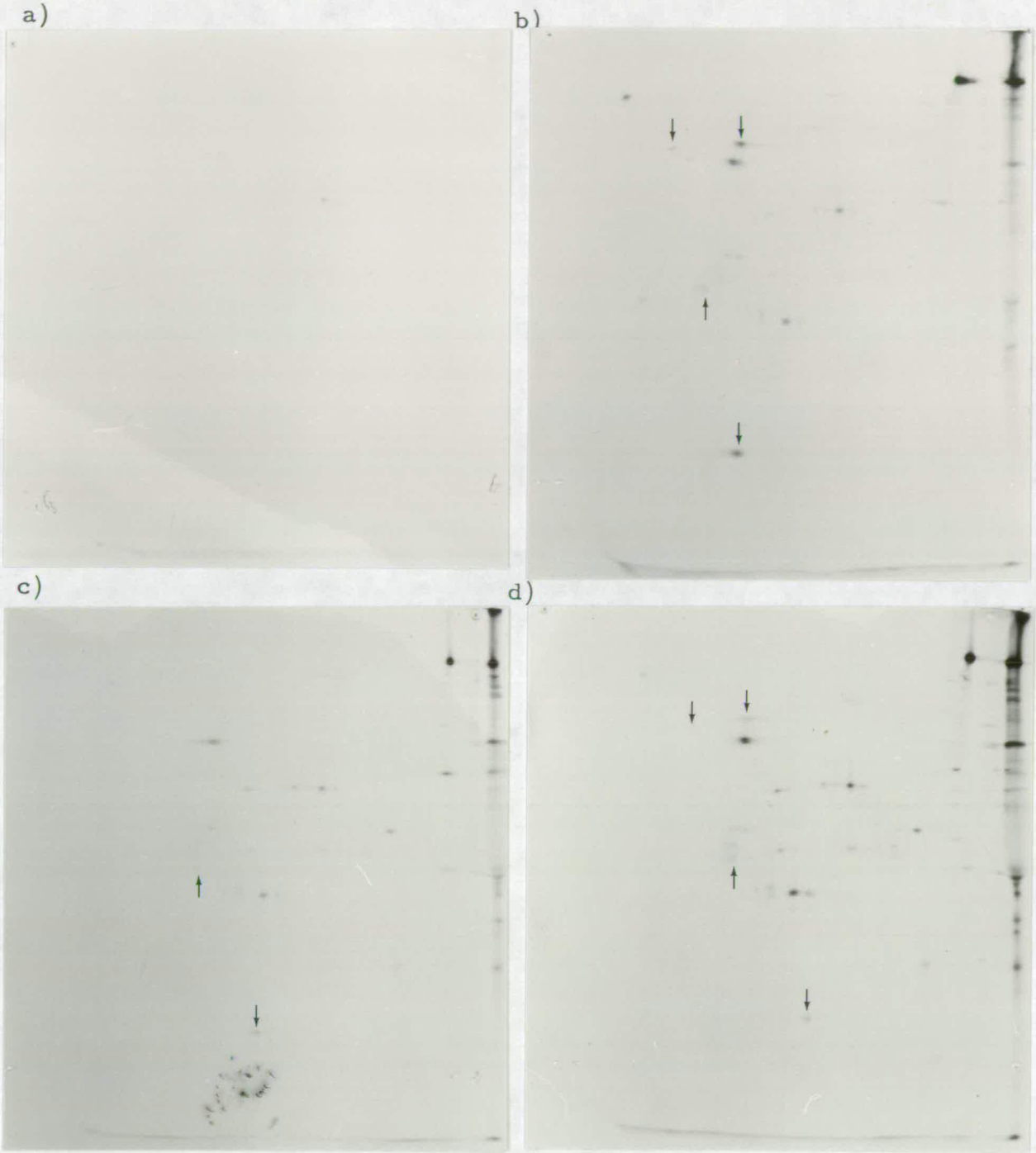


Fig. 8.3 2D gels of the immunoprecipitates shown in fig. 8.2. a) control serum/SK17, b) Thai serum/SK17, c) Thai serum/T9 c94 and d) Thai serum/T9 c96. Differences between b), c) and d) are arrowed. The probable identities of the immunoprecipitated proteins are shown in fig. 8.4.

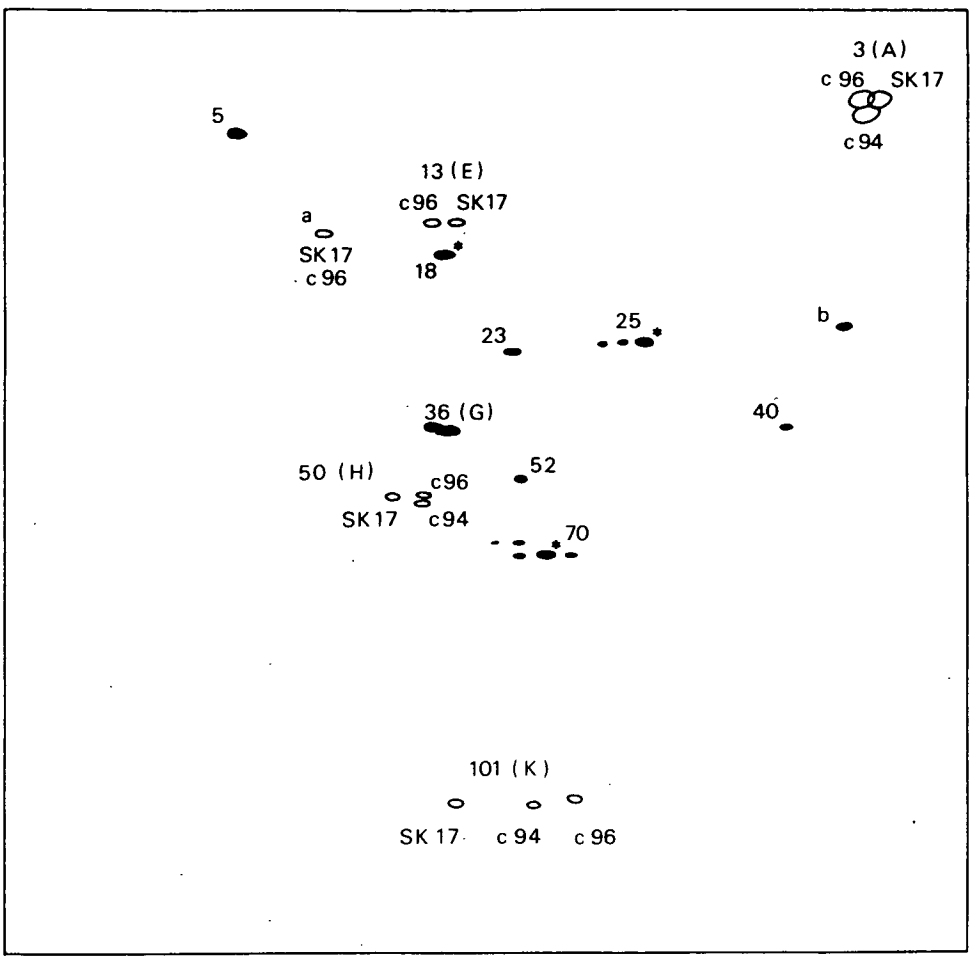


Fig. 8.4 Diagram detailing the proteins immunoprecipitated by Thai human serum from extracts of SK17, T9 c94 and c96 (see fig. 8.3).

● , proteins immunoprecipitated from all three extracts;

○ , those precipitated only from one culture as indicated;

* , proteins weakly precipitated by control serum.

was the dominant antigen recognised. In addition, two other proteins (a and b, fig. 8.4 b) were immunoprecipitated which could not be correlated with any previously characterised proteins. Most of the major components correspond to bands resolved on one-dimensional gels (figs. 8.1 and 8.2) as detailed in Table 8.1. However, some strongly-labelled bands seen on the one-dimensional gels, e.g. those of 162, 38 and 33 kd, could not be correlated with proteins detected on the two-dimensional gels. This suggests that the two methods of analysis are detecting slightly different sets of proteins. The stage of synthesis and other properties of the antigens identified on the two-dimensional gels are collated in Table 8.1. Four of these antigens are synthesised predominantly by schizonts (#3, 13, 50 and 101); five are preferentially lost during saponin lysis of parasitised erythrocytes (#5, 13, 23, 25 and 36); and one is glycosylated (#3). In addition, five have already been shown to vary electrophoretically between isolates (#3(A), 13(E), 36(G), 50(H) and 101(K)). Hence, it was of interest to compare the proteins immunoprecipitated by the same batch of Thai serum from extracts of the T9 clones 94 and 96 which have different electrophoretic variants of several proteins (see Chapter 5). Autoradiographs of the proteins precipitated from these two lines are shown in fig. 8.3 c) and d) and the results of this comparison are summarised in fig. 8.4. The strain-invariant proteins - #5, 18, 23, 25, 40 and 70 - were all precipitated from SK17 and both T9 clones as was protein b. The relative amount of each protein precipitated from the three

Table 8.1 Parasite antigens identified on 2D gels

Protein #	Immunoprecipitated from: Control	SK17	T9c94	c96[i]	Major, reproducible	Present in saponin supernatant	Stage of synthesis[ii]	Strain variation	Glycosylated	band on 1D gels[iii]
3		+	+	+	+		S	A	+	190kd
5		+	+	+	+	+	I			148?
13		+		+	+	+	S	E		
18	(+)	+	+	+	+		I			92
23		+	+	+	+	+	I			
25	(+)	+	+	+	+	+	I			72
36		+	+	+	+	+	I	G		
40		+	+	+			I			
50		+	+	+			S	H		
70	(+)	+	+	+	+		I			42
101		+	+	+	+		S	K		27
a[iv]		+		+						
b[iv]		+	+	+						

(i) See figs. 8.3 & 8.4. A single sample of Thai human serum was used throughout.
(ii) S = synthesised predominantly by schizonts; I = synthesised throughout cycle.
(iii) See fig. 8.2.
(iv) a and b were not positively identified with any previously characterised proteins.

strains did differ slightly, e.g. #40 and b were more intensely labelled in the T9 precipitates than in that of SK17. In the case of the strain-variant proteins #3(A), 50(H), 101(K) and possibly 36 (G), proteins of molecular weight and isoelectric point corresponding to the expected electrophoretic variants were detected in both clones. In all three strains, protein #3 was the major component immunoprecipitated. Variant protein #13(E) was precipitated from clone 96 in the expected position but was not detectably precipitated from clone 94 which has a different electrophoretic variant of #13 from both clone 96 and SK17. Other examples of proteins apparently being immunoprecipitated from only one or two of these three strains are indicated in figs. 8.3 and 8.4. These results and their significance are discussed in section 8.5.

8.4 Target Antigens of Two Monoclonal Antibodies

An alternative approach to the identification and characterisation of parasite antigens is to use hybridoma technology to produce monoclonal antibodies directed against them (Perrin et al, 1980; McBride et al, 1982). The target antigens can then be identified biochemically by immunoprecipitation techniques such as those described in section 8.2 or by affinity chromatography. The latter method was adopted by Dr. R. Hall (Molecular Biology Dept., Edinburgh University) to purify the antigens recognised by two different monoclonal antibodies raised against proteins of the Thai

isolate K1. The properties of these monoclonal antibodies - 2.2 and 7.3 - have been described in detail by McBride et al (1982) and Hall et al (1983, 1984b). Each monoclonal gave different immunofluorescent staining patterns and each reacted with different sets of parasite strains and stages. However, both were thought to be directed against merozoite surface components and both precipitated proteins of approximately 190 kd as analysed on one-dimensional SDS-acrylamide gels. In view of the accumulating evidence that similar, high molecular weight antigens may be involved in inducing protective immunity against other species of malaria parasite (Boyle et al, 1982; Freeman and Holder, 1983a), it was of considerable interest to determine a) whether these two monoclonal antibodies were directed against the same or different antigens and b) to identify these target antigens on two-dimensional gels and to compare them with the proteins recognised by immune human serum.

As outlined in section 2.8, each monoclonal antibody was covalently linked to sepharose and then used to affinity purify the antigens from a ^{35}S-methionine labelled extract of K1. Samples of the sepharose-bound proteins provided by Dr. Hall were solubilised and then analysed by two-dimensional gel electrophoresis. Both antigen preparations were electrophoresed separately and as a mixture. It was found that both monoclonals bound identical proteins: one major, high molecular weight, basic polypeptide and a number of minor, smaller polypeptides (fig. 8.5). The gels were calibrated by co-electrophoresis of the labelled antigen with an

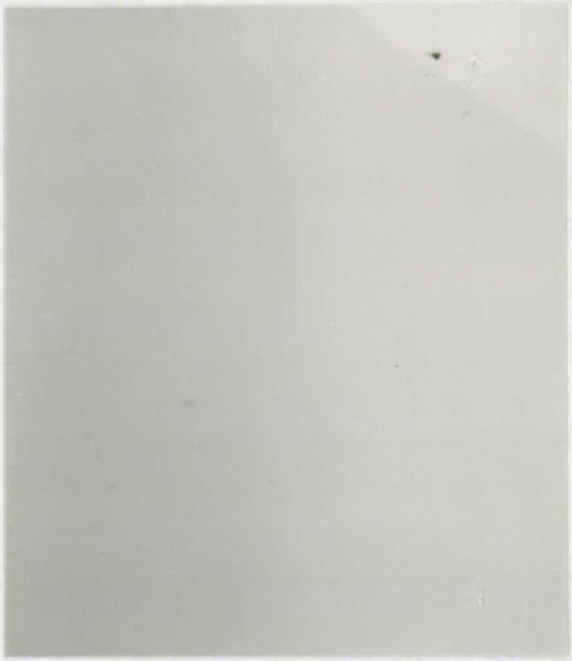


Fig. 8.5 Major and minor components of a ^{35}S-methionine labelled extract of K1 bound by sepharose-linked monoclonal antibody 7.3.

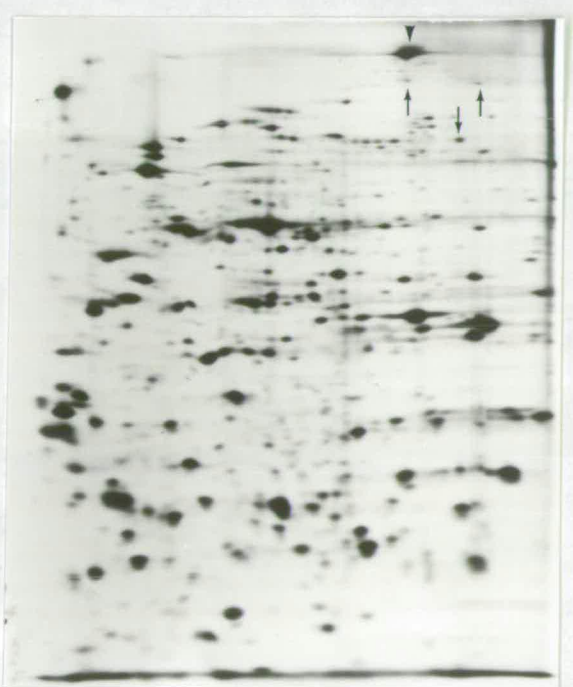


Fig. 8.6 ^{35}S-methionine labelled extract of T9 c32 indicating variant A4 of protein #3 (▾) and the three minor, basic polypeptides (↑) equivalent to those recognised by monoclonal antibody 7.3 in K1 (see fig. 8.5).

unlabelled extract of K1. Silver staining was used to visualise all the major parasite proteins for purposes of internally standardising the gel and then direct autoradiography was used to detect the labelled antigens. This allowed the major 190 kd protein recognised by these monoclonal antibodies to be identified as protein #3. This protein has already been shown to be a major parasite product which is synthesised predominantly by schizonts, varies electrophoretically between strains and is also glycosylated. The minor proteins detected in the antigen preparations, included three basic polypeptides of approximately 150, 149 and 110 kd. Although these had not been previously characterised, all three were clearly identifiable on two-dimensional gels of whole parasite extracts of strains possessing the same electrophoretic variant of protein #3 (A4) as K1 (fig. 8.6). In addition, both monoclonals bound a 28 kd protein which was identified as #101 and a 50 kd protein equivalent to #50 was detected at very low intensity on some autoradiographic exposures. Proteins #50 and #101 are also synthesised predominantly by schizonts and vary electrophoretically between isolates. Both monoclonal antibodies have already been shown to immunoprecipitate a series of lower molecular weight polypeptides in addition to the major 190 kd antigen (Hall et al, 1983, 1984b). Therefore it seems likely that the minor components detected on the two-dimensional gels are also specifically recognised by the monoclonals and they may, for example, be proteolytic products of the 190 kd protein #3.

8.5 Discussion

Numerous studies have used one-dimensional gel electrophoresis to analyse the proteins recognised by sera from endemic areas (e.g. Kilejian, 1980a; Reese et al, 1981; Brown et al, 1982a; Myler et al, 1982; Perrin and Dayal, 1982). A diversity of parasite isolates, ways of extracting antigens, sources and types of sera and methods of immunoprecipitation used makes detailed comparisons of these studies difficult. In addition, the heterogeneity, in terms of titre and specificities of anti-falciparum antibodies, between serum samples, is well established (Collins et al, 1971; Reese et al, 1981; Brown et al, 1983a). Despite these natural and experimental sources of variation, some common features are evident in these one-dimensional gel studies. Several major parasite antigens are high molecular weight proteins (95-200 kd) which are synthesised predominantly by schizonts (Perrin et al, 1981a; Brown et al, 1982a; Myler et al, 1982). 4-6 major antigens in this size range, including the dominant 190 kd protein can be seen in figs. 8.1 and 8.2 and previous experiments (see fig. 6.4) suggest that most are synthesised by mature parasites. However, not all antigens are schizont-specific. Two dominant stage-invariant antigens of 70-76 kd and 40-45 kd are frequently immunoprecipitated (e.g. Myler et al, 1982) and these probably correspond to the 75 and 42 or 44 kd bands seen in figs. 8.1 and 8.2. Many other antigens can also be precipitated by human serum. However, the difficulties in making accurate

comparisons between different one-dimensional gel studies inevitably mean that much potentially useful information is being lost or overlooked. Hence, one major benefit of analysing parasite antigens by two-dimensional gel electrophoresis will be the ability to unambiguously identify many antigens, including minor ones, and to make meaningful comparisons between different sets of results.

The two-dimensional gels in section 8.3 showed that the sample of Thai serum analysed immunoprecipitated several parasite proteins including #3*, 5*, 13, 18, 23, 25, 36, 40, 50*, 70 and 101*. More extensive two-dimensional studies have been published by Brown et al (1982 b, 1983 a and 1983 b) on antigens recognised by sera from Papua New Guinea (PNG). A comparison shows that at least eight of the above proteins (those underlined) were also precipitated by PNG sera. Some PNG sera strongly precipitated proteins not detected by the Thai serum, e.g. proteins #8*, 11*, 35*, 47* and 49. As concluded from 1D gel studies, a high proportion of immunoprecipitated proteins are specific to mature parasites. At least 50% of the antigens identified on 2D gels were synthesised predominantly by schizonts, including those indicated above with an asterisk. Another significant trend is that several of the immunoprecipitated proteins were among those selectively depleted by saponin lysis of parasitised erythrocytes. These include proteins # 5, 8, 11, 13, 25, 35 and 36 (Table 6.2). It is feasible that proteins lost during saponin lysis may, perhaps by virtue of their subcellular location, also be major components released into the

bloodstream during merozoite release *in vivo* and may therefore come in direct contact with the host's immune system. In this context, it is of relevance that proteins #8, 11, 13 and 36 have also been noted to vary electrophoretically between isolates.

There is a large amount of evidence showing that parasite isolates differ antigenically from each other (see section 1.5). This is also demonstrated in section 8.3, where a single sample of Thai serum immunoprecipitated slightly different sets of parasite proteins from three different strains. The strain-specific precipitation of antigens has also been demonstrated by Brown *et al* (1983 b) by two-dimensional gel analysis. There are two reasons why proteins might be immunoprecipitated from some parasite strains but not from others. One is that these proteins may only be present in some isolates. This was found to be the case for the strain-specific antigens detected by Brown *et al* and the same may apply to some of the strain-specific differences apparent in figs. 8.2 and 8.3. The second explanation is that, although the homologous protein is present in all strains, different antigenic variants exist and only certain variants can be recognised by some sera. It seems likely that this may be true for protein #13(E) which was precipitated from SK17 and clone 96, but not from clone 94 by the same batch of Thai serum. This protein also varies electrophoretically between these three strains and variant 2 (clone 94) may therefore differ antigenically from variants 1 (SK17) and 3 (clone 96). Electrophoretic variants of other proteins -

#3 (A1, 2 and 4), #36 (G1 and 2), #50 (H1, 3 and 6) and #101 (K1, 3 and 6) - were all recognised by the Thai serum (see fig. 8.4). It is probable that these electrophoretic variants share at least some common antigenic determinants recognised by this serum. Another possibility is that the variants also differ antigenically, but the serum contains antibodies directed against each antigenic type.

One of the drawbacks of studying parasite proteins by two-dimensional gel electrophoresis is that in most cases the biological function and properties of the resolved polypeptides is not known. Hence it was of considerable interest to identify the target antigens of monoclonal antibodies 2.2 and 7.3 on two-dimensional gels. The results show that, despite having different properties, both monoclonal antibodies were directed against the same parasite antigens. The major target was protein #3 which was also the dominant antigen recognised by the Thai human serum. Indirect immunofluorescence tests have demonstrated that McAb 7.3 reacts specifically with schizonts and merozoites but only with those of certain parasite isolates. McAb 2.2, in contrast, reacts with all the stages of the erythrocytic cycle and with all isolates screened (McBride et al, 1982; Hall et al, 1983). This difference in strain specificity between the two monoclonals implies that the antigen has both common and strain-specific epitopes and that McAb 7.3 must be directed against the latter. Since the 190 kd antigen (protein #3) is only synthesised by schizonts and is lost during reinvasion (Hall et al, 1984b; pers. obs.) the reaction of McAb 2.2 with all the erythrocytic

stages is more difficult to explain. The most likely explanation is that the antigenic determinant defined by McAb 7.3 is either lost or altered during invasion but that part of the 190 kd protein bearing the 2.2 epitope is conserved during invasion and is detectable in ring and trophozoite-infected erythrocytes. The 190 kd antigen (protein #3) is known to be processed during schizogony (Hall et al, 1984b) and is almost certainly identical to the schizont-specific 195 kd protein described by Holder and Freeman (1982). They also demonstrated that this protein is processed late in schizogony to produce a major merozoite surface protein of 83 kd (Freeman and Holder, 1983b). This is thought to be processed further during or after reinvasion to a 60 kd protein. Intermediates of the 195→83 kd conversion have molecular weights of 153, 150 and 110 kd and these may correspond to the minor 150, 149 and 110 kd proteins recognised by McAbs 2.2 and 7.3 on the two-dimensional gels (fig. 8.5). No proteins equivalent to the 83 kd and 60 kd products could be identified on these gels. Instead, two proteins of approximately 50 and 28 kd (# 50 and 101) were detected. These are both labelled almost exclusively during the development of schizonts (Chapter 6) and were largely lost or degraded during reinvasion (personal observation). Thus, it is more likely that these may be small fragments produced during the 195→83 kd processing rather than later cleavage products of the 83 kd merozoite protein. The molecular relationships between proteins #3, 50 and 101 could be elaborated by pulse-chase labelling experiments or by peptide mapping of the three polypeptides.

Combining the specificity of monoclonal antibodies with the high resolving power of 2D gel electrophoresis is obviously of great potential for identifying and characterising parasite antigens. However, 2DGE is equally valuable for analysing the complex mixtures of antigens immunoprecipitated by polyspecific sera. Although the work presented in this chapter was based on only a single sample of human serum it has allowed the identification of several parasite proteins precipitated by serum from endemic areas and of some which may vary antigenically between isolates. A more extensive screening of serum samples using a range of parasite isolates would clearly produce a more detailed analysis of antigens recognised by the host immune system and might also provide some insight into the extent and significance of the antigenic diversity within P. falciparum.

DISCUSSION

9.1 Introduction

The results presented in this thesis raise a number of general questions: firstly, the relative merits of two-dimensional gel electrophoresis (2DGE), enzyme typing and antigenic analysis for strain differentiation in P. falciparum (as discussed in section 9.2); secondly, the relevance of the intraspecific variation detected by these techniques for the parasite's population structure (section 9.3); thirdly, the validity of using in vitro cultures for the analysis of P. falciparum (section 9.4); fourthly, the molecular and genetic basis of the protein variation detected by 2DGE in P. falciparum and in other organisms (sections 9.5 and 9.6); fifthly, the identity and biological functions of the parasite proteins resolved by 2DGE (section 9.7); and finally, the value of 2D gel analysis in malaria research (section 9.8).

A number of other points, namely the technical aspects of radiolabelling parasite proteins and specific aspects of the analysis of strain- and stage-specific proteins and parasite glycoproteins and antigens, have been discussed in some detail in earlier chapters and will not be considered further here.

9.2 A Comparison of Strain-typing Techniques

Ideally, a good strain-typing system should possess several features including i) a large enough number of variable characters and/or sufficient polymorphism within them to permit strains to be

"fingerprinted", ii) the sensitivity to detect minor components in samples of mixed parasite genotypes, and iii) the system should be technically straightforward, reproducible and easily standardised. It is clear from the results presented in Chapter 4 that the 2D gel analysis of parasite proteins is a very powerful technique for characterising isolates of P. falciparum. It does not have all the attributes of an ideal system - both the preparation and the analysis of the 2D gels are technically demanding and time-consuming procedures. However, 2D gel electrophoresis does have advantages over the methods of enzyme electrophoresis and antigen analysis which have also been used for strain differentiation (section 1.5). The major advantages are that a large number of parasite products can be screened on a single 2D gel and that a high level of heterogeneity occurs amongst them. For example, 18% of approximately 100 polypeptides analysed in Chapter 4 varied in one or more of the isolates examined. Of the 13 polypeptides used for strain typing, seven (A, B, C, D, E, H and K) were classed as having four or more electrophoretic variants. In the case of another set of seven polypeptides (A, B, D, G, H, K and L) the commonest electrophoretic variant of each was present in less than two-thirds of the isolates screened. This degree of heterogeneity was sufficient to discriminate uniquely between all the Thai and Gambian isolates analysed. Six Papua New Guinean isolates, although apparently identical to each other, were also distinct from these other isolates. (See Table 4.2). In comparison, enzyme typing (as outlined in section 1.5) has been

based on six different enzymes: only two or three variant forms of each have been identified and only in three cases (GPI, ADA and LDH) does the dominant enzyme variant occur at a frequency of less than 90%. Thus, analysis of these enzymes is only of limited scope for strain differentiation in P. falciparum.

Serotyping samples of P. falciparum - either on the basis of their S-antigens or other antigenic specificities - is potentially of greater value. More than eighteen different S-antigen specificities have been detected (Wilson et al, 1969) and the stable, reproducible nature of their expression could allow the development of a useful strain-typing method (Wilson, 1980). The production of monoclonal antibodies against different strains of parasite has also revealed considerable antigenic diversity in P. falciparum (McBride et al, 1982, 1984; Knowles et al, 1984). McBride et al (1982) characterised 27 long term cultures on the basis of their immunofluorescence reactions with 14 monoclonal antibodies. 18 different combinations of antigenic specificities were identified making this system of similar power to 2DGE for strain characterisation. Since 14 of the isolates analysed by 2DGE (see Chapter 4) were also typed by McBride et al (1982) these two systems can be compared directly. All 14 isolates could be uniquely defined by their protein profiles on 2D gels. However, antigen typing was less discriminating: only 11 different combinations of antigen specificities were found. No antigenic differences were detected between isolates K1 and T22 and none among isolates K28, K29 and T17 whereas between four

and eight differences were detected on 2D gels. The monoclonal antibodies used by McBride et al were raised against two different isolates of P. falciparum. By using a wider range of isolates to generate monoclonals of new specificities it should be possible to increase even further the power of this method of strain typing.

An important attribute of a strain typing system is the ability to identify samples or cultures which are not pure strains but contain a mixture of parasite genotypes. The extent to which mixed infections occur in vivo has implications for the immunology of the disease and the biology of the parasite. It is also critical to have defined, homogeneous, cloned lines of parasite available for basic research purposes. The methods of strain-typing discussed here, differ in their ability to detect minor parasite types in mixed samples. Typing parasites using monoclonal antibodies in indirect immunofluorescence tests is extremely sensitive. Since individual parasites can be screened microscopically, it is possible to detect low levels ($< 0.1\%$) of a positively reacting genotype in a largely negative parasite population. However, in the case of both enzyme typing and 2D gel analysis, minor components will only be detected if the absolute amount of a variant enzyme or protein in the sample analysed is high enough to be visualised by the staining or autoradiographic system used. Under standard conditions it is thus the proportion of the minor genotype(s) in the parasite population which is critical. It has been estimated, by using indirect immunofluorescence to determine the proportion of different antigenic types in a culture,

that minor genotypes must make up approximately 5% of the parasite population in order to be reliably detected by both enzyme electrophoresis and 2D gel techniques (McBride, pers. comm.).

When the practical aspects of these methods are considered, it is clear that both enzyme typing and antigenic analysis have advantages over 2DGE in that they are rapid, relatively simple techniques and give results which are easily analysed. 2DGE is, in contrast, a more lengthy and complicated procedure and the final gels or autoradiographs are more difficult to analyse. 2DGE of metabolically labelled proteins does, however, require only relatively small amounts of cultured material and allows parasite proteins to be specifically detected by autoradiography. This is in contrast with enzyme typing which requires larger amounts of parasite material and in which the differentiation of parasite and host cell enzymes may be a problem.

For purposes of typing isolates of P. falciparum it is clear that 2D gel analysis and antigen typing methods are both superior to enzyme electrophoresis in terms of the amount of intraspecific variation revealed. Serotyping parasites with monoclonal antibodies is of similar power to 2D gel analysis for strain differentiation but one potential drawback of antigen typing is that it appears to be based on a small set of immunodominant parasite products. 22 monoclonal antibodies produced by McBride (1984) are thought to be directed against five independent classes of antigen as determined by their strain specificities and by immunochemical studies. The polypeptides

shown to vary electrophoretically on 2D gels, on the other hand, are likely to be more representative of the parasite's genotype.

This comparison of enzyme typing, antigenic analysis and 2DGE emphasises the fact that these techniques have different relative merits in terms of the amount of intraspecific variation detected, the sensitivity of the technique and the practical aspects of screening isolates. Thus, although antigenic analysis and 2DGE are superior to enzyme electrophoresis in many respects, the method of choice for characterising isolates will largely be determined by the conditions under which the work is to be done and on the use to which the results are to be put.

9.3 Geographical Variation within P. falciparum

One application of strain typing data is to look for evidence for the existence of distinct geographical races of P. falciparum. This has been an area of interest in malaria research for over 50 years. Early work was based on in vivo observations of the severity of the infections caused by different strains of parasite, their response to antimalarial drugs and their infectivity to mosquitos. After studying over 700 patients infected with different strains of P. falciparum originating from India, Africa and Europe, Shute and Maryon (1951) were left in no doubt that geographical differences did exist. For example, Italian strains caused more severe infections, were less susceptible to quinine and produced higher gametocyte densities than did Indian or African strains. Only the Italian strains were infective

to Anopheles atroparvus, a species of mosquito found in Europe, whereas Indian and African parasites were not. In contrast, strains from the same continent had similar properties.

As reviewed in section 1.5, recent work based on molecular characters of the malaria parasite has produced a different picture. In many cases, the same enzyme types, antigenic specificities and protein variants have been found in parasites from widely separated areas. In addition, surveys of parasite antigens (Wilson et al, 1969; Knowles et al, 1984) and proteins (Tait, 1981; Chapter 4) have revealed considerable heterogeneity among strains originating from the same region. These results have been interpreted as evidence that P. falciparum forms a single, interbreeding population throughout its range. However, it must be pointed out that there have been few large scale surveys of parasites from different areas and no strong evidence against the alternative hypothesis that distinct, geographically separated parasite populations may exist. The most extensive studies have been of enzyme variation in parasites from parts of Africa and S. E. Asia (Carter and McGregor, 1973; Carter and Voller, 1975; Sanderson et al, 1981; Thaithong et al, 1981; Myint-Oo et al, 1984). The results suggest that the parasite populations in both continents are generally very similar. For example, the same variants of three enzymes (GPI, ADA and PEP) occurred at very similar frequencies in parasites from the Gambia (W. Africa) and from Thailand. However, some qualitative and quantitative differences have been detected. For example, a rare form of GPI (GPI-3) has

only been found in isolates from Thailand and Burma. Furthermore, whereas all Thai isolates typed have had the same variant of LDH (LDH-1) an additional variant (LDH-2) has been found at a low frequency among Burmese isolates and at a higher frequency in Gambian samples. Although not conclusive, these differences imply that some degree of geographical variation may exist both within and between these two continents.

In view of the greater intraspecific diversity detected by antigen typing and by 2D gel analysis, these techniques would be expected to provide enough information to resolve this question of regional differences. However, with the exception of studies by Wilson et al (1969) on S-antigens in the Gambia and by Knowles et al (1984) on variant antigens in Papua New Guinea, the numbers of isolates screened for antigenic differences have been relatively small. In line with enzyme typing data, the same antigenic specificities or phenotypes can be found in isolates from different parts of the world (McBride et al, 1982) and at present there is little evidence for regional variation. However, it is possible that more extensive surveys of parasite populations will reveal differences in the frequency of particular antigens. Knowles et al (1984) noted that one particular antigenic specificity, previously detected in only two-thirds of a panel of culture-adapted isolates of diverse origin (McBride et al, 1982) was ubiquitous in the Papua New Guinean isolates tested and suggested that this might be a characteristic of parasites from this region.

The analysis of protein variation by 2DGE has demonstrated considerable heterogeneity among isolates collected from the same region (Tait, 1981; Chapter 4). Although only a small number of isolates have been analysed - 7 by Tait (1981) and 21 in this thesis - both studies have suggested that detectable regional differences may be superimposed on this basic pattern of intraspecific diversity. The evidence indicates that such regional variation may be found both within S. E. Asia and between it and Africa. As detailed in sections 4. 5 and 4. 6, the variants of some proteins did not appear to be randomly distributed among isolates from three different regions of Thailand. In particular, proteins A, B, D and K had variants which were specific to, or predominant in, isolates from a single region. In addition, consistent differences were seen between Gambian and S. E. Asian isolates. Tait (1981) found that two Gambian isolates, FMG and G2, had unique variants of four (AT # 1, 3, 4 and 34) out of fourteen strain-variant proteins analysed. Similarly, among the thirteen proteins used for strain typing in this thesis, the Gambian isolate, G1, had unique variants of three proteins (B, C and G) and variants of four others (D, E, K and M) which were relatively rare in the Thai isolates (see Table 4. 2). It is noteworthy that both studies identified possible Gambian-specific variants of the same parasite proteins. As detailed in Table 4. 3, protein AT # 1 corresponds to protein B and AT # 3 and 4 to C in this thesis. However, since protein B (AT # 1) is "hypervariable" the occurrence of specific variants in this small

sample of Gambian isolates is not necessarily indicative of geographical differences. As detailed in Chapter 4, the same pattern of regional variation can also be seen by making quantitative comparisons between isolates. Fig. 4.4 shows the number of proteins which differ in all possible pairwise comparisons of the isolates typed in Chapter 4. These figures clearly demonstrate the high degree of intraspecific diversity detected in P. falciparum by 2DGE. This heterogeneity is evident within all three regions of Thailand. However, the isolates from Songhkla and Kanchanaburi have on average more protein variants in common among themselves and between regions, than do the isolates from the Tak region. As proposed in Chapter 4, the greater heterogeneity of the Tak isolates may be due to the diverse origins of the infections from which these cultures were derived - T17 from Burma and T19 and T22 from Cambodia. Although this explanation presupposes the existence of geographical differences within S.E. Asia there is evidence from enzyme typing studies (Thaithong et al, 1981; Myint-Oo et al, 1984) to show that Burmese isolates may also have some enzyme variants not seen in Thai isolates. Looking at more widely separated regions, fig. 4.4 shows that the protein profile of the Papua New Guinea isolates is very similar to many of the Thai isolates - especially those from Songhkla and Kanchanaburi. However, in marked contrast, the Gambian isolate (G1) had consistently low numbers of variants in common with the Thai isolates. Although much larger numbers of isolates from a range of different regions would need to be analysed in order to draw any definite

conclusions, the results of these 2D gel studies indicate that some degree of geographical separation may be present in P. falciparum.

To conclude, the analysis of P. falciparum at the molecular level, particularly by enzyme typing and antigenic analysis, has suggested that this parasite species is generally very similar throughout its range. The 2D gel analysis of protein variation has, however, given a preliminary indication that distinct, geographically separated parasite populations may exist. If this finding is substantiated by further work, it would appear that these three techniques differ in their ability to detect such geographical variation. This might be explained by these methods screening distinct classes of parasite products which differ in the extent and pattern of their intraspecific variation. It can also be postulated that the discrepancy between molecular studies and clinical observations in detecting major differences between parasites from various parts of the world is due to the type of character analysed. For instance, in comparison with electrophoretic enzyme variation, the course of the infection in vivo and the infectivity of the parasites to mosquito species may be more closely related to environmental conditions (e.g. duration of transmission season and species of vector mosquitos present) and may thus be more likely to exhibit geographical variation.

9.4 The Use of in vitro Cultures for the Analysis of P. falciparum

One point which should be considered here is that much research on strain variation in P. falciparum, including all the work presented

in this thesis, has been based on cultured parasites. The development of the in vitro culture system was of great value for such studies, allowing, for instance, parasites to be maintained indefinitely, cryopreserved for future reference or cloned to produce pure lines. The widespread use of culture-derived parasites makes it necessary to ask whether these parasites differ from those occurring in vivo. Electron microscopy has shown that parasites grown in vitro are morphologically similar to those in natural infections (Langreth et al, 1978). However, some parasite properties have been reported to change following their establishment in vitro including the formation of knobs on the surface of the host erythrocyte (Langreth et al, 1979) and the ability to produce gametocytes (Ponnudurai et al, 1982). Thus, it is possible that the synthesis of some proteins and the expression of some antigens, may not be seen in all cultured parasites. However, metabolic labelling studies have suggested that parasites from long-term cultures do synthesise a very similar array of proteins to those of recently isolated parasites (Boyle et al, 1983 b). Thus, changes in parasite phenotype caused by in vitro culture conditions are unlikely to be a major problem for purposes of typing isolates, particularly if stable characters are selected for analysis. More important is the question of whether parasites established as in vitro cultures are representative of the parasite populations from which they were derived. It is possible that some parasite genotypes may not be able to adapt to in vitro conditions. There is evidence that some strains will grow faster than others in vitro and that this can

result in one genotype outgrowing another in originally heterogeneous isolates (Rosario, 1981; Chapter 5). Thus, it is likely that long term cultivation will select for adaptable, fast growing parasites and will select against uncompetitive, slow growing genotypes. This may affect estimates of intraspecific diversity and comparisons between parasite populations. One way round this problem is to use parasites as soon as possible after collection from an infected patient. Another is to clone parasites from recent isolates in order to establish minor or slow-growing genotypes in vitro and to make a more diverse and representative collection of parasites available for research purposes.

Another problem related to the use of long-term cultures, particularly where several strains are maintained in the same laboratory, is the possibility of cultures being accidently mislabelled or contaminated with parasites of another strain. In the latter case the contaminating strain may outgrow the original parasites and become the dominant genotype in the culture. This has long been recognised to be a problem in the cultivation of mammalian cell lines in vitro where many cases of intra- and interspecies contamination have been documented (Nelson-Rees and Flandermeyer, 1977). In the case of P. falciparum, it has been found that cross-contamination of cultures can take place if a rigorous culture technique is not adopted (pers. obs.). This possibility has serious implications for research based on cultured parasites. Some findings reported in the literature, for example changes in the drug

sensitivity (LeBras et al, 1983) and protein profiles (Brown et al, 1983b) of parasites during in vitro culture, may be explained by such cross-contamination. As discussed in section 5.4, this is also the probable cause of the gradual appearance of a second genotype of parasite in certain cloned lines derived from the T9 isolate. In addition, where several isolates grown in the same laboratory are found to be identical, e.g. the Papua New Guinea isolates analysed antigenically by Schofield et al (1982) and other PNG isolates screened in this thesis (Chapter 4), then the possibility that one type of parasite has contaminated and outgrown those in the original cultures cannot be discounted. The identity and origin of cultured parasites is of particular importance in investigations of intra-specific diversity and the population structure of P. falciparum. A number of isolates screened during the course of this thesis had identical protein profiles and several appeared to be the same as T9 clone 94. These included two isolates from Thailand (K31 and T21), one from Sri Lanka (SL3) and three from the Gambia (FMG, 62/52 and BW). These isolates were excluded from the results reported in Chapter 4 because it is probable that they arose as a consequence of poor culturing technique and subsequent cross-contamination. An alternative hypothesis that this genotype actually exists as a distinct, reproductively isolated strain of P. falciparum in these three widely separated regions, seems less likely.

All the above observations underline the need for developing sensitive methods for the routine typing of isolates; for monitoring

the identity of parasites cultured in vitro; and for establishing reference collections, or strain banks, of well characterised isolates of P. falciparum.

9.5 Protein Variation in P. falciparum: Its Molecular and Genetic Basis

The molecular and genetic basis of the protein variation detected by 2DGE is of considerable interest. Some knowledge of the underlying causes of the observed variation is necessary for correctly interpreting the protein profiles of different isolates, identifying cultures of mixed genotypes and assessing the genetic diversity within the species. As detailed in Chapter 4, several types of strain-specific variation were seen on 2D gels. These included the presence of proteins in certain isolates which were absent, or not detectable, in others. Also, in some cases, proteins were metabolically labelled to varying extents in different strains. However, the commonest type of variation was the substitution of a given protein in one isolate by one of a different electrophoretic mobility in another isolate. In most instances these have been assumed to be electrophoretic variants of the same protein. These different types of intraspecific protein variation are considered in more detail below in relation to their possible molecular origins.

Presence/absence differences. Examples of proteins which were seen only in extracts of certain isolates are illustrated in figs. 4.5 - 4.10 and 5.1 - 5.4. These included proteins #16 (Appendix 1), 112 and 127 (Appendix 2) and a number of otherwise uncharacterised

proteins. Such differences may have a sound genetic basis but could also arise in other ways. For instance, it is necessary to rule out artifactual differences. Different procedures or reagents used during sample preparation could affect the final protein pattern; different culture conditions may induce or suppress the synthesis of specific proteins; and the detection of stage-specific proteins will be dependent on the age-distribution of parasites in each culture. Some spurious intraspecific variation is bound to be detected if only a limited analysis of each parasite strain is performed and this may include some of the presence/absence differences listed above. However, highly reproducible examples of this type of variation were found in comparisons of the three parasite strains - isolate SK17, T9 clone 94 and T9 clone 96 - which were extensively analysed during the course of this thesis (fig. 5.4). These differences presumably must reflect genotypic or stable phenotypic variation between isolates. In some cases, the absence of particular proteins has been shown to have a genetic basis in other organisms. 2D gel analysis of mouse liver samples by Elliot (1979) showed that two proteins which were not detected in one mouse line but were found in another, were present in reduced amounts in the heterozygote. It is also possible that the expression of certain genes may be lost during adaptation to in vitro growth or during long term cultivation. The loss of specific antigens has been noted in short term cultures of the primate malaria P. knowlesi (Butcher, 1979) and the loss of the knobs and their associated histidine-rich knob protein has been

reported in P. falciparum (Kilejian, 1979). It is of interest in this respect, that among the T9 clones, those of the clone 96 genotype were knobby, while those of the clone 94 type were knobless (Thaithong et al, 1984). It is possible that the absence of certain proteins in clone 94 might be correlated with the loss of the erythrocyte knobs.

Variation in labelling intensity. 2 proteins (#107 and #123) appeared to be labelled to different extents among the isolates screened. Examples of this can be seen in figs. 4.5 - 4.10. This type of variation may only be a less extreme form of the presence/absence kind and the possible explanations given for the latter are equally applicable here. It has not been established how reproducible this variation is in the isolates examined here but similar and reproducible differences in intensity have been observed in other 2D gel studies (Howard et al, 1983) and thus may have a genetic basis. The rate of synthesis of a protein could, for example, be directly affected by changes in the nucleotide sequence of the gene itself or its mRNA transcript. The intensity of labelling will also be dependent on the rate of degradation of the protein and this in turn may be affected by changes in the structure or conformation of the native molecule.

Electrophoretic shifts. Where apparently homologous proteins differ in electrophoretic mobility between isolates, two patterns of variation can be discerned. In the simplest case, two or three variant forms of a protein have been identified and these differ in isoelectric point

(pI) only. In the second type a more complex pattern of variation is seen, often with a greater number of possible variants which differ in their apparent molecular weight (MW) and in their pI. In view of probable differences in their molecular basis these two patterns of variation are considered separately:

i) pI shifts. Proteins I, J, L and M used for strain typing (fig. 4.3) and some additional proteins (# 119 and # 133) all showed this type of variation. Shifts in pI have also been the most common type of genetic variation detected in other organisms by 2DGE (Elliot, 1979; Lee et al, 1979; Leigh Brown and Langley, 1979; Racine and Langley, 1980; Rosenblum et al, 1984). This is the classical type of variation expected from point mutations in structural genes where the substitution of one amino acid for another in the protein product may result in a charge change. However, post-translational modifications such as sialylation, phosphorylation or amidation, could also produce such charge changes and differences between strains could be due to genetically determined changes in the primary protein structure removing or creating modification sites. It is probable that protein F, which has three variant forms, is subject to some kind of modification. As detailed in section 4.3, isolates with the more basic variants invariably also have low levels of the more acidic ones. This suggests that the basic variants are derived from an acidic precursor, small amounts of which remain in an unmodified or partially modified form. A similar type of precursor-product relationship has been noted in other 2D gel studies (e.g. Elliot, 1979).

ii) pI and/or MW shifts. It is of interest that most of the electrophoretic variation detected in P. falciparum falls into this category (see fig. 4.3). In some cases, e.g. proteins A, C, E and G used for strain typing, only three or four variants of each protein were seen. In others, e.g. proteins B, D, H and K, there were five or more different variants and a more complex pattern of variation. These different electrophoretic forms have been assumed to be genetic variants of the same protein. Genetically determined differences in MW have also been noted by Rosenblum et al (1983, 1984) and these may arise in several ways. Since many proteins undergo some type of post-translational processing, it is likely that this could play a major role in producing MW differences. For example, where proteolytic processing takes place, proteins of different MW could be the result of changes in the amino acid sequence which alter the specific sites of cleavage in the precursors of different variants. Large or small MW differences could be produced in this way and where processing also removes acidic or basic amino acids then changes in pI might also be expected. Another common type of post-translational modification is glycosylation. Again, the presence or absence of suitable sites of glycosylation in different protein variants would affect the extent of glycosylation and may cause changes in the apparent MW and/or pI of the modified protein. This has been shown to be the case for MW plus pI differences between allelic variants of certain proteins in other systems (Anderson and Anderson, 1979). It may be significant that

variant protein A (#3) is a major parasite glycoprotein and that protein #6 which also varies in both MW and pI is glycosylated (Chapter 8). In all instances where strain-specific variation is due to differential processing or modification, this could either be specified by the primary structure of the protein itself and thus reflect variation in the structural gene or it could instead be the result of variation in the enzymes of the processing system (Cheney et al, 1984).

Another possible source of MW variation is in the coding region of the structural gene itself and there are some known examples of both a gene and its protein product being polymorphic in size (Manning and Gage, 1980; Muskavitch and Hogness, 1982; Hudspeth et al, 1984). This phenomenon may be more likely to occur in genes containing repetitive nucleotide sequences where unequal crossing-over could generate size differences. Thus it is of interest that two P. falciparum proteins which are known or thought to harbour amino acid sequence repeats - the S-antigens (Coppel et al, 1983) and the histidine-rich knob protein (Wallach et al, 1984) - also exhibit size polymorphism (Wilson and Ling, 1979; Anders et al, 1983; Leech et al, 1984b).

Among the proteins exhibiting strain-specific variation, protein B is unique. Extremely large differences in both MW and pI were observed among the putative variants of this protein. In addition, variation was noted in the intensity of labelling of this protein in some isolates while in others no variant of B was identified.

The same pattern of variation was observed for this protein by Tait (1981) and as discussed in section 9.7 it is likely that protein B variants are equivalent to the diverse group of S-antigens described by Wilson and Ling (1979). However, the relationship between these variants and the molecular and genetic basis of their variation remains unclear.

In order to correctly interpret any pattern of electrophoretic variation observed on 2D gels it is necessary to determine a) that putative variants are variants of the same protein rather than products of unrelated genes and b) whether variants are products of different alleles of a gene and/or the result of post-translational processing or modifications. Techniques such as peptide mapping of purified proteins or immunochemical characterisation with specific antisera or monoclonal antibodies, can be used to confirm the identity of protein variants. In theory, the genetic basis of the variation can be studied by conventional genetic analysis. However, making crosses between different lines of P. falciparum is not yet practicable. Instead, information about the genetic basis of variation has been inferred from the distribution of variants in isolates and cloned lines of the parasite. Since the asexual, erythrocytic parasites are haploid each parasite will express only one allele of each gene. Thus, where variants occur independently in different isolates it may be assumed that they are the products of different alleles. Conversely, if allelic variants occur together in the same

isolate then this implies that more than one genotype of parasite is present in that isolate. However, where post-translational processing is involved, partial modification could result in more than one "variant" polypeptide being seen even in a cloned line. It is not possible to rule out this possibility where a specific variant has only been observed in combination with other variants of the same protein (see Chapter 4). A different consequence of post-translational processing is that apparently unrelated proteins, of very different MW and pI, could be derived from the same structural gene. Proteins H and K used for strain typing appeared to be related in this way. Although seven variants of K were identified and five of H, only eight different combinations of H and K variants were seen. Comparisons showed a marked correlation between the size and pI of variants of H and those of K (fig. 9.1). Since variants of K (26.7-28.0 kd) are much smaller than those of H (47.0-48.6 kd) it is very probable that K is a proteolytic product of H. No other correlations were found between variants of different polypeptides.

9.6 The Extent of the Intraspecific Variation Detected by Two-dimensional Gel Analysis

Although only 13 strain-variant polypeptides detected on 2D gels were used for typing P. falciparum isolates (Chapter 4), several other polypeptides were also observed to vary electrophoretically in the isolates screened in this thesis (section 4.3, Appendix 2). 25 out of a total of 110 polypeptides (i.e. 23%)exhibited some type of strain variation. In the similar 2D gel study by Tait (1981) 14 of 35 major

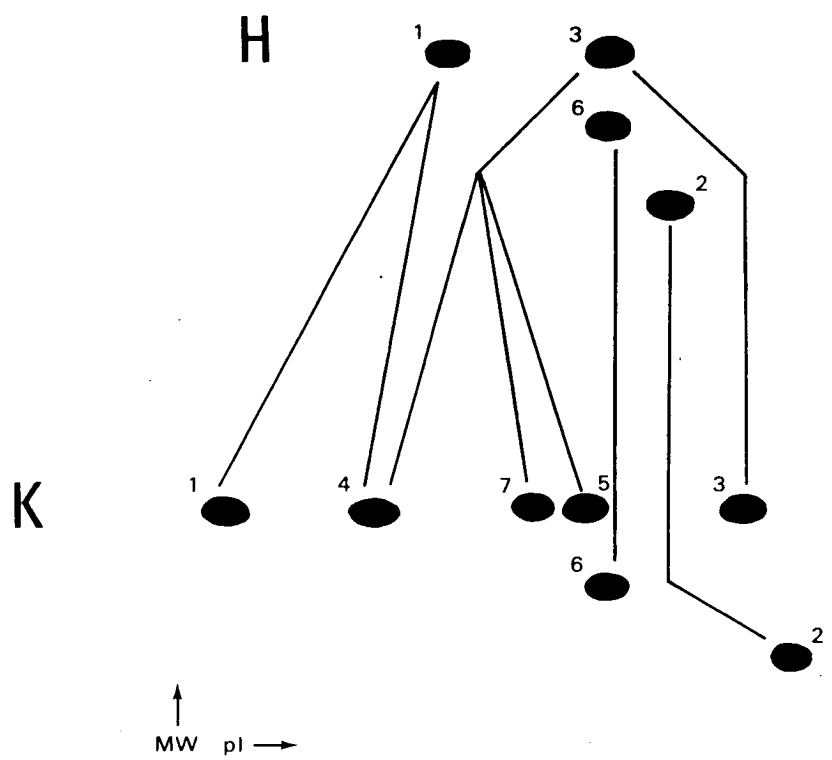


Fig. 9.1 Diagram showing correlation between the MW and pI of variants of H and those of K occurring in the same isolate.

parasite polypeptides (40%) varied between isolates. Most of the variant polypeptides identified by Tait were also found to vary among the isolates examined in this thesis (Table 4.3). As discussed in section 4.6, this discrepancy between the proportions of variant proteins in the two studies is mainly due to a disproportionately large amount of the variation occurring amongst more abundant parasite proteins. However, in both studies, a much higher degree of intraspecific variation was apparent than has been found by 2D gel analysis in other organisms (Table 9.1). Both the percentage of proteins (or loci) which were polymorphic and the extent of the heterogeneity (or heterozygosity, in the case of diploid organisms) at each locus appear to be significantly greater in P. falciparum than in man, mouse, or Drosophila.

Much more variation has been detected in P. falciparum by 2D gel analysis than by enzyme electrophoresis (section 9.2). This too, is in contrast with studies of intraspecific variation in other systems. Previously, where 2DGE has been used to assess genetic variation, it has invariably detected much lower levels of variation than conventional 1D gel techniques (McConkey et al, 1979; Leigh Brown and Langley, 1979; Aquadro and Avise, 1981; Ohnishi et al, 1982). Two explanations for this difference have been proposed (Edwards and Hopkinson, 1980). One is that 2DGE is not as sensitive to protein variation as 1D gel systems are. The other is that 2D gel analysis is biassed towards abundant cellular proteins which may be under greater selective constraints and hence

Table 9.1 Estimates of genetic variation in P.falciparum and other organisms by 2D gel electrophoresis

Species	Tissue/cell type (# analysed)	% of proteins or loci polymorphic (total screened)	Average heterozygosity (or heterogeneity)(ii)	Reference
Man	Fibroblast cell lines (5)	1.2% (300)	0.006	Walton et al, 1979
	Kidney (25)	0% (83)	0	Smith et al, 1980
	Brain (145)	1.1% (176)	0.0002	Comings, 1982
	Lymphocytes (28)	10.2% (186)	0.024	Goldman & Merril, 1983
	Erythrocyte lysates (27)	8.7% (46)	0.031	Rosenblum et al, 1984
Mus musculus	Kidney	3.5% (85)	0.02	Racine & Langley, 1980
Drosophila melanogaster	Whole flies (20)	11.1% (54)	0.04	Leigh-Brown & Langley, 1979
P.falciparum	Erythrocytic parasites (21)	18% (100)(i)	0.09	Chapter 4 & Appendices

(i),(ii) - see notes (i),(ii), Table 9.2

intrinsically less variable than the soluble enzymes used for isozyme studies. Both these factors also have a bearing on the amount of intraspecific variation detectable in P. falciparum by 2DGE. There is clear evidence that this technique detects a more limited range of genetic variation than 1D methods (Wanner et al, 1982; McLellan et al, 1983). In the O'Farrell 2D system, isoelectric focusing (IEF) is carried out under denaturing conditions and thus only proteins which differ in net charge can be resolved on the first dimension gels. In contrast, 1D systems run under non-denaturing conditions are sensitive to much more subtle differences in protein structure. Protein variants having different amino acid substitutions which produce the same nominal charge change can often be resolved, presumably due to their differential affects on the conformation, ionisation or other properties of the native polypeptide. Thus, it is inevitable that a certain amount of genetic variation will remain undetected by 2DGE. The class of protein analysed is, however, perhaps the more important factor in determining the extent of variation detectable by a given technique. In the case of enzyme electrophoresis and 2D gel electrophoresis, the hypothesis is that the soluble enzymes screened by the former are intrinsically more variable than the abundant cellular proteins detected on 2D gels. This is probably an oversimplified view since even among polymorphic enzymes, groups with different levels of heterogeneity have been identified and in some cases, these have exhibited similar levels of variation to those of proteins resolved on 2D gels (Leigh

Brown and Langley, 1979). Correlations have been found between the extent of genetic variation and the structural and functional characteristics of different classes of protein. Soluble enzymes, for example, can be classified according to the origin and diversity of their substrates. Gillespie and Langley (1974) defined Group I enzymes as having unique substrates, usually generated and used intracellularly and Group II enzymes as those with multiple substrates often of extracellular origin. The latter group have consistently shown higher levels of variability than Group I enzymes in studies of diverse groups of organisms (Gillespie and Langley, 1974). Similarly, higher levels of variation have been noted in enzymes with a regulatory role as compared to non-regulatory enzymes (Johnson, 1974) and in monomeric as opposed to multimeric enzymes (Harris et al, 1977). In the latter case, the requirement for interactions between subunits is assumed to impose greater restrictions on the structure of the protein and thus reduce its genetic heterogeneity. The same proposal has been put forward to account for the comparatively low levels of variation found in several 2D gel studies (McConkey, 1982). The abundant, cellular proteins detected on 2D gels are likely to include a high proportion of structural or membrane components and, in view of the organisational complexity of biological membranes, these components would be expected to be less variable than soluble proteins or enzymes.

The concept that classes of proteins with different biological roles may have different levels of genetic variability is also relevant

to the intraspecific variation detected in P. falciparum by different techniques. In the case of enzyme electrophoresis it may be relevant that, with the exception of PEP, all the enzymes used for strain typing, fall into the less variable Group I category. This may partly explain why relatively little intraspecific variation was revealed by enzyme analysis. In comparison, a relatively large amount of variation was detected in P. falciparum by 2DGE and a high proportion of this variation occurred amongst the most intensely labelled (and therefore the most abundantly synthesised) proteins which is in contrast with the situation found in other organisms by 2D gel analysis (McConkey et al 1979). It is of interest that the variation detected in P. falciparum can be divided into two classes on the basis of the stage-specificity of the proteins involved. As shown in Table 9.2, proteins synthesised late in the asexual cycle (during the late trophozoite and schizont stages) have a higher proportion of polymorphic proteins with, on average, more variants per protein and a greater degree of heterogeneity in the population than do proteins synthesised throughout the cycle. This observation suggests a possible explanation for the large amount of variation detected in the abundant parasite proteins. Schizont-specific polypeptides include the major components of the merozoite which, as the only extracellular stage of the asexual cycle, is a prime target for the host's immune system (section 1.3). It is to be expected that the parasite as a species will be under strong selective pressure to maintain a diversity of merozoite antigens in the population. Thus,

Table 9.2 Comparison of the variation detected in stage-specific and stage-invariant proteins of P.falciparum

Class of protein[iii] (# screened[i])	% polymorphic	Ave. # of variants/ polymorphic protein	Ave. heterogeneity[ii] for: polymorphic proteins	all proteins
Stage-invariant (67)	16%	2.5	0.40	0.07
Trophozoite +/or schizont specific (19)	32%	5.3	0.65	0.20

(i) Analysis was based on the set of major, reproducible parasite proteins defined in Section 3.4. Only proteins whose variants showed altered electrophoretic mobility were classed as polymorphic.

(ii) In order to compare the extent of variation in the proteins of the haploid malaria parasite with that given by heterozygosity estimates for diploid organisms, the frequencies of different variants of a parasite protein were used to calculate the probability that two randomly selected genotypes would have different variants of that protein. This 'heterogeneity' was calculated as = $1-(fvar1)^2-(fvar2)^2-.....-(fvarN)^2$. (This is the same formula as is used to determine expected heterozygosity in diploids.) The total heterogeneity among the polymorphic parasite proteins was then averaged over all the proteins screened. For purposes of estimating the frequencies of the variants, the six identical Papua New Guinea cultures have been assumed to be derived from a single isolate.

(iii) As defined in Table 6.1.

it is possible that the abundant parasite proteins seen on 2D gels are not typical, highly conserved, structural components but instead include a set of important and variable parasite antigens. This possibility is backed up by the fact that several of the abundant, schizont-specific polypeptides are recognised by serum from endemic areas (Chapter 8; Kilejian, 1980a; Perrin and Dayal, 1982; Brown et al, 1982a, 1983a) and by the great intraspecific diversity detected among schizonts and merozoites by antigenic analysis (McBride et al, 1982).

9.7 The Identity of the Proteins Resolved by Two-dimensional Gel Electrophoresis

As demonstrated by the results presented in this thesis, 2DGE is a very powerful technique for analysing the proteins of P. falciparum The approach used in most of this work has been to separate and characterise the parasite proteins in terms of various physical and biological properties (e.g. pI, MW, strain-distribution, stage-specificity) but without any direct reference to their identity. In this way it has been possible to build up a considerable bank of data about the proteins of the parasite and to apply this information to specific research problems - namely the extent of intraspecific variation and the development of a strain typing system for P. falciparum. However, in order to realise the full potential of this analytical technique in malaria research, it is necessary to be able to determine the identity and biological function of the proteins resolved by 2DGE. There are several ways in which particular spots detected on 2D gels

can be equated with specific proteins. The most direct method is to purify the protein in question: its identity on 2D gels can then be determined by co-electrophoresis of the purified sample with an unfractionated parasite extract. Affinity chromatography is of particular value in this respect. Monoclonal antibodies have been used both in this thesis (Chapter 8) and in other studies (Pearson and Anderson, 1983) to purify and identify specific antigens on 2D gels. A similar approach using substrates, co-factors or lectins, for example, could be employed to prepare particular parasite enzymes or glycoproteins for analysis. Alternatively, it may be possible to exploit these specific reagents in order to identify parasite proteins directly on 2D gels after electrophoresis of crude extracts e.g. by immunoblotting procedures. Where neither of these approaches is feasible an indirect method is to correlate changes in protein profiles with differences in the properties of various parasite samples analysed. In this way it may be possible, for example, to identify specific protein variants with different enzyme types. In fact, apart from a few well characterised parasite antigens, little is known about the identity of the proteins resolved on 2D gels. The aim of this section is to review what is known about some of these proteins and what can be deduced about others.

a) Parasite antigens

For obvious reasons the identification of parasite antigens is a major area of research. A number of the polypeptides resolved

by 2D gel electrophoresis have been shown in this thesis (Chapter 8) and in other studies (Brown et al, 1982b; 1983a) to be recognised by serum from endemic areas. These include protein # 1 (B), 2, 3 (A), 5 (E), 8 (C), 11, 13 (E), 18, 22, 23, 25, 36, 40, 47, 49, 50 (H), 70 and 101 (K). Many of these proteins are major biosynthetic products of the erythrocytic parasites; several are synthesised predominantly by schizonts; and a high proportion vary electrophoretically between strains. However, in most cases, little else is known about the identity, function or immunological relevance of these antigens. Some studies have tried to correlate the immunoprecipitation of specific proteins with the protective properties of different serum samples (Brown et al, 1982b, 1983b). In this way immunoprecipitation of a major 96 kd polypeptide (Pf 96, Brown et al, 1982b), equivalent to protein #18 in this thesis, has been associated with the inhibition of parasite growth in vitro by some human sera. Two parasite antigens which have been positively identified on the 2D gels are the p190 of Hall et al (1983) and the S-antigens of Wilson et al (1969). These correspond to proteins #3 and 1 respectively and since they have been extensively characterised by other techniques they will be considered in some detail below.

The p190 antigen. As reported in Chapter 8, protein #3 was found to be the main target of two monoclonal antibodies, 2.2 and 7.3, described by McBride et al (1982) and Hall et al (1983, 1984b). 2D gel analysis showed that this antigen is a major parasite glycoprotein

synthesised predominantly by late trophozoites and schizonts which varies electrophoretically between strains. p190 is also identical to the 195 kd antigen characterised by Holder and Freeman (1982). The detailed analysis that has been carried out on this antigen (Hall et al, 1983, 1984b; Holder and Freeman, 1982, 1984; Freeman and Holder, 1983b) has revealed that this high MW schizont antigen is processed during the final stages of schizogony to produce discrete products of approximately 83, 42 and 19 kd which are present on the surface of merozoites and which are recognised by immune serum. Peptide mapping has indicated that the 83 and 42 kd polypeptides are non-overlapping fragments of the p190 precursor. Heidrich et al (1984) have reported that the 190 kd glycoprotein is processed into three glycosylated products of 53, 38.5 and 15 kd found in isolated merozoites and it is probable that the 38.5 and 15 kd glycoproteins are equivalent to the 42 and 19 kd merozoite surface antigens described above.

Although protein #3 is a major biosynthetic product of P. falciparum cultures no amino-acid labelled proteins corresponding to its processed products could be unambiguously identified on 2D gels. Presumably these proteins are only very minor components of the labelled parasite extracts analysed. However, in addition to glycosylated protein #3 (gp 1), metabolic labelling with ^{3}H-glucosamine identified three strongly labelled glycoproteins, gp 4, 5 and 7 (figs. 7.2 - 7.4), of approximately 50, 44 and 37 kd respectively. These probably correspond to three of the glycoproteins identified

in merozoites by Heidrich et al (1984) and this leads to the conclusion that the 50 and 37 kd glycoproteins (gp 4 and 7) resolved by 2DGE (see Chapter 7) may be breakdown products of protein #3. Both these glycosylated proteins appeared to correspond to schizont-specific components of amino-acid labelled parasites (proteins #49 and 74). Both monoclonal antibody 2.2 and 7.3 reacted with proteins #50 (H) and #101 (K) which are relatively major products of labelled schizonts. It is not clear how these are related to protein #3. They may be proteolytic fragments derived from protein #3 prior to the specific production of the merozoite antigens. Alternatively, they may be unrelated to p190 and perhaps co-purify during immunoadsorption. It should be possible to resolve this question by, for example, peptide mapping.

The intraspecific diversity which exists in this one parasite protein has been well documented. Its electrophoretic variability has been detailed in this thesis (Chapter 4) and by Newbold et al (1984). A number of monoclonal antibodies directed against the p190 protein react with restricted sets of parasite isolates and it has been possible to identify eleven different "serotypes" of this molecule on the basis of its reaction with a panel of such antibodies (McBride, 1984). By comparing the isolates typed by 2DGE in this thesis (Chapter 4) with those typed with monoclonal antibodies by McBride et al (1982) it has been possible to correlate one electrophoretic variant of protein #3 (A used for strain typing) with the specificities of some of these antibodies. All the isolates characterised by variant A4

reacted positively with the monoclonals 6.1, 7.3 and 7.6, whereas isolates with different variants did not. Some monoclonal antibodies against this molecule reacted with all isolates tested, implying that p190 has both conserved and variable regions. Although the molecular basis of the antigenic diversity is not known, it is clear that some of the strain-specific antigenic determinants, reside in the amino acid structure of the protein, rather than in its carbohydrate component (Hall et al, 1984b). Peptide mapping of different variant proteins has demonstrated considerable polymorphism in the primary structure of the protein (Newbold et al, 1984).

The p190 precursor and its products have been shown to be recognised by human serum from a variety of endemic regions and sources (Brown et al, 1983b; Hall et al, 1984b; Holder and Freeman, 1984) and there is both indirect and direct evidence implicating them as targets of protective immune responses in vivo. As discussed by Newbold (1984) homologous high molecular weight antigens with similar properties to that of P. falciparum have been identified in other species of malaria parasite. In the case of P. yoelii and P. chabaudi, two rodent malaria species, either passive or active immunisation against this high molecular weight antigen resulted in some degree of protective immunity. In P. falciparum monoclonal antibodies against parasite components of 82 and 41 kd inhibit parasite growth in vitro (Perrin and Dayal, 1982) and purified p190 has been shown to induce some degree of protective immunity in primates (Hall et al, 1984a; Perrin et al, 1984a, b). Thus, at

present it appears that this set of parasite antigens are very good candidates for trial vaccines.

S-antigens. As discussed in section 9.5, protein #1 (B) showed a much more extreme type of intraspecific variation than did other variant proteins screened by 2DGE. At least ten different types of protein B were identified among the 21 isolates typed in Chapter 4 and these variants differed widely in both MW and pI. The same pattern of variation was also noted in this protein (AT #1) by Tait (1981). The extreme heterogeneity in this high molecular weight, acidic protein suggested that protein B might be equivalent to the equally diverse, heat-stable, soluble S-antigens described by Wilson et al (1969). The S-antigens have also been shown to vary electrophoretically over a similar range of MW and pI (Wilson and Ling 1979; Winchell et al, 1984). This hypothesis has recently been confirmed by the work of Anders et al (1983) which identified the S-antigen of four Papua New Guinea (PNG) isolates on 2D gels. This antigen migrated as a 220 kd protein with a pI of approximately 4.2. Comparison of Anders' 2D gels with those of one of the same PNG isolates (FCQ2) analysed in this thesis showed clearly that the PNG S-antigen was identical to variant B1. This variant was detected in all the PNG isolates screened in Chapter 4 and also in the Thai isolate, SK17.

Until recently, little could be said about the molecular and genetic basis of the diversity observed in this class of parasite antigen. However, Coppel et al (1983) have cloned part of the PNG

S-antigen gene. Analysis has revealed that much of this S-antigen is made up of an eleven amino acid repeating sequence and that the antigenic specificity resides in this repetitive structure. In line with the lack of cross reactions between different S-antigens, hybridisation experiments using DNA probes have failed to detect homologous sequences in the genome of parasites expressing heterologous S-antigens. Thus, it is possible that the S-antigens are the products of a family of structurally distinct genes, only one of which is present and expressed in each parasite line. The presence of unrelated repetitive sequences in different S-antigens might be expected to produce proteins of very different amino acid composition and this may be one explanation for the variation observed in the intensity of labelling of protein B in different isolates.

2D gel analysis of synchronous cultures (see Chapter 6) showed that protein B is synthesised predominantly by developing schizonts and is probably among the proteins depleted by saponin lysis of parasitised cells (Table 6.2). Both these findings fit with the known properties of the S-antigens. Antibodies directed against S-antigens give characteristic patterns of immunofluorescence showing the same stage-specificity, i.e. no reaction with ring forms but bright staining with schizonts. Staining may be localised in the parasitophorous vacuole and a zone of extracellular fluorescence can be seen around schizonts as might be predicted for a secreted parasite antigen (Coppel et al, 1983; Saul et al, 1984).

Although significant amounts of S-antigen can be found in the

sera of some infected individuals, very little is known about the biological function of these parasite products. A monoclonal antibody directed against the S-antigen of certain PNG isolates has been reported to inhibit the in vitro growth of these isolates (Saul et al, 1984). These antigens may therefore be implicated in the development of protective immunity.

b) Other parasite proteins

A number of other parasite proteins and antigens have been the subject of recent research (WHO, 1984b). These include the histidine-rich knob protein (KP) which is associated with the formation of "knobs" on the surface of infected erythrocytes (Kilejian, 1979, 1984; Hadley et al, 1983; Leech et al, 1984b; Vernot-Hernandez and Heidrich, 1984); surface antigens involved in the strain-specific attachment of schizont-infected erythrocytes to endothelial cells (Leech et al, 1984a); and glycophorin-binding proteins which play a role in the invasion of uninfected host cells (Jungery et al, 1983; Perkins, 1984). These have all been well characterised in terms of their biochemical and biological properties. The KP, for example, is known to be synthesised by ring stage parasites and inserted into the erythrocyte membrane later in development as a 75-92 kd polypeptide; it is synthesised by knobby (K^+) but not by knobless (K^-) clones; and shows molecular weight variation between isolates. However, despite such information it has not been possible to correlate any of the above with proteins

analysed on 2D gels. Similarly, although the GPI enzyme types of several isolates screened in this thesis were known (Thaithong, pers. comm.; Walliker, pers. comm.) no good correlation could be drawn between any of the 2D protein variants and this parasite enzyme. Another variant enzyme, LDH, is known to be a major parasite product of 35 kd (Hyde et al, 1984). It is possible that protein #75, an intensely labelled parasite protein of this molecular weight, may be this enzyme. Although no electrophoretic variation was seen in protein #75 in this thesis, a more basic variant has been observed on 2D gels of certain isolates (Fenton, pers. comm.).

Although only a small number of the proteins resolved on 2D gels have been positively or tentatively identified, it is clear that such information can be of great use in malaria research. The high resolving power of the 2D gel system means that once a specific spot on a gel is identified as a particular parasite protein, then other properties of that protein, e.g. its stage-specificity, intracellular location or antigenicity, can be determined directly by analysis of unfractionated parasite extracts. In addition, as demonstrated in this thesis, the identification of antigens recognised by monoclonal antibodies on 2D gels is of value both for characterising the antibodies themselves and for investigating the molecular and biological properties of their target antigens.

9.8 Two-dimensional Gel Electrophoresis and Malaria Research

2DGE was first used to investigate intraspecific variation in *P. falciparum* by Tait (1981). This thesis has extended 2D gel analysis to a wider range of parasite proteins and a larger number of parasite isolates and the results presented here in relation to protein variation within this species have largely confirmed those of Tait. Considerable diversity has been demonstrated among the parasite proteins and sufficient variation detected to develop a strain-typing system of high sensitivity. In theory, more than one million different combinations of strain-restricted protein variants may exist within the parasite population and the probability of two parasite isolates exhibiting the same protein profile by chance is less than one in four thousand. However, 2DGE is not an ideal strain-typing method. As discussed in section 9.2, it is a lengthy, technically demanding technique and analysis of the resulting gels may not be a simple matter. There are two ways in which 2DGE, as a strain-typing method, could be developed. One is to incorporate more sophisticated techniques, e.g. multiple gel electrophoresis systems (Anderson and Anderson, 1978a,b) and computer-aided gel analysis (Anderson *et al*, 1981), to enable large numbers of samples to be compared more easily and to extend analysis to a wider range of the parasite proteins. The other approach would be to simplify the procedure and to make it more practicable. For example, silver staining could be used in place of autoradiography to detect the major

parasite proteins and strain-typing could be restricted to proteins whose electrophoretic variants are easily distinguished without recourse to complex double-labelling procedures.

One obvious application of such a strain-typing system is to investigate the question of regional variation within the species. The limited strain comparisons carried out by Tait (1981) and in this thesis (Chapter 4) have indicated that some degree of geographical variation both within and between continents may exist. However, larger scale screening of parasites from a number of different regions is required to confirm or reject this finding.

2DGE also has other possible applications. One practical objective is to build up a protein map for the malaria parasite and to use it to collate as much information as possible about the biochemical, biological and immunological properties of these parasite products. Some steps have been made in this thesis towards this by characterising the major proteins in terms of their strain specificity (Chapter 4), stage-specificity during the asexual cycle (Chapter 6), glycosylation (Chapter 7) and antigenicity (Chapter 8). However, it is clear that much more analysis could be done. It would be of value to follow the synthesis and fate of parasite proteins during the erythrocytic cycle in more detail than was done in Chapter 6; to identify specific subsets of proteins, e.g. merozoite components; and, as discussed in section 9.7, to characterise more of the proteins resolved on 2D gels in terms of their biological functions. 2D gel analysis will be of great value

for analysing the products of genetic crossing experiments, when these become feasible, since the products of a large number of parasite genes may be screened simultaneously. Another obvious extension of 2DGE is the analysis of other stages of the parasite's life cycle such as the sporozoites, their replication within hepatic cells and the development of gametocytes within erythrocytes (section 1.3). The identification of common and stage-specific proteins or antigens may be relevant to the biology of the parasite and the control of the disease.

Although there may be scope for improving some of the technical aspects of the 2D gel system and the need for extending analysis to more parasite proteins and more parasite isolates, the results presented in this thesis clearly demonstrate the great potential which 2DGE has for malaria research.

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Appendix 1 Table characterising the major, reproducible proteins of isolate SK17[i]

Protein #	AT[ii] #	MW (kd)	pI[iii]	Strain[iv] variation	Stage-specificity[v] & other properties
1(B)	1	200.2	<4.5	pI/MW (10+)	S *
2		201.6	4.8		gp
3(A)		196	>6.6 (0)	pI/MW (4)	S gp ag
5		150.4	4.5		* ag
8(C)	3/4	122.6	5.6 (-15a)	pI/MW (4)	S *
9		119.5	5.9 (-11b)		
10(D)		116.9	6.3 (-5a)	pI/MW (6)	
12		113.7	5.5 (-16a)		
13(E)	6	107.2	5.3 (-18a)	pI/MW (4)	S * ag
15		103	5.4 (-18b)		
16		106	5.9 (-11b)	+/-	R
18	8	96.8	5.3 (-19b)		ag
19	7	98	5.7 (-13a)		
22	9	72.8	5.5 (-16a)		
23	10	72.2	5.5 (-16b)		*? ag
25	12	73.2	5.9 (-11b)		* ag
28		71.2	6.1 (-8a)		
29		71.7	6.2 (-7b)		
30		72.9	6.4 (-3a)		
31(F)		69.7	6.1 (-9b)	pI (3)	
32		66.0	5.9 (-11b)		
33		66.1	6.0 (-10b)		
34		64.6	5.0		
36(G)	14	56.9	5.3 (-18b)	pI/MW (3)	* ag
37		60.4	5.7 (-14a)		
38		59.8	5.9 (-11b)		
39		56.9	6.1 (-8b)		
40		58.5	6.3 (-6b)		ag
42		57.6	6.5 (-2a)		
43		57.1	>6.6 (-1b)		
44		54.0	5.9 (-10a)		
45		53.4	6.1 (-9b)		
46		53.5	6.1 (-8b)		
47	15/16	52.9	5.2 (-20)		S
49	20	51.2	4.9		S gp
50(H)	21	48.4	5.1	pI/MW (5)	S ag
51		51.2	5.5 (-16a)		S
52	18	51.9	5.5 (-16b)		T,S
53	19	51.9	5.9 (-12b)		
54	17	51.9	5.9 (-11b)		
55		48.3	6.2 (-7a)		
56		48.3	6.4 (-4b)		
57		48.4	6.5 (-2b)		
58		46.1	6.5 (-2b)		S
59		46.1	>6.6 (-1b)		
60		44.5	6.6 (-1b)		
62		45.4	5.4 (-16a)		S
63		45.4	5.5 (-16b)		S

Protein #	AT #	MW	pI	strain variation	Stage-specificity & Other properties
66		40.7	4.7	pI/MW (5)	R
69		43.1	5.5 (-16b)		
70	22	43.1	5.6 (-15b)		ag
71	23	43.2	5.7 (-14b)		
72	24	43.4	5.8 (-12a)		
73	25	43.4	5.9 (-11b)		
75	28	36.4	5.8 (-13b)		
76		37.9	6.5 (-2b)		
77		36.9	6.5 (-2b)		
78	26	37.7	4.8		*
79		36.3	4.7		
81	29	35.2	4.6		
83		34.3	5.0		
84		34.0	5.2 (-20)		R
85(I)		34.1	5.4 (-17a)	pI (2)	
86(J)		33.2	5.5 (-16a)	pI (2)	
90		34.0	6.5 (-3b)		S
91		33.8	6.5 (-1a)		
92		32.3	<4.5		
93		31.6	5.9 (-10a)		
94		32.4	6.3 (-5b)		R
95		32.6	6.4 (-4b)		
96		29.5	4.8		
97		29.9	5.2 (-20)		
98(M)	31	29.8	5.5 (-16a)	pI (2)	
99		28.7	5.1 (-21)		T,S
100	32	27.7	5.2 (-20)		
101(K)		27.9	5.4 (-18b)	pI/MW (7)	S ag
102		26.6	5.3 (-19b)	pI/MW (3)	
103	33	26.4	5.5 (-16a)		
105		26.7	5.9 (-11a)		
106		26.7	6.0 (-10b)		*
107		26.6	6.1 (-8b)		S
108		27.6	6.2 (-7a)		
109		27.5	6.3 (-6b)		
111		29.0	6.5 (-2a)		
113		27.0	6.4 (-3b)		
116		26.6	6.6 (-1b)		
117	35	25.1	5.2 (-19a)		
118		25.6	5.9 (-11a)		S
119		25.5	6.0 (-10b)	pI (2)	
120		23.7	5.3 (-18a)		
121		23.1	5.0		
122		23.8	5.8 (-12b)		
123		23.9	5.9 (-10a)		
124		24.2	6.1 (-9b)	pI/MW (2)	
125		24.2	6.3 (-7b)		S
126		24.8	6.3 (-5b)		
128		25.3	6.4 (-4b)		T,S
129(L)		24.5	6.4 (-4b)	pI (2)	*
130		24.8	6.4 (-3b)		
133		21.3	5.8 (-13b)	pI (2)	

(i) as defined in Figs. 3.10 & 3.11.
(ii)-(v) see corresponding notes to Appendix 2.

Appendix 2 Table summarising the properties of other parasite proteins refered to in Chapters 3 - 9[i]

Protein #	AT[ii] #	MW (kd)	pI[iii]	Strain[iv] variation	Stage-specificity[v] & other properties
6		141.3	6.4 (-4a)	pI/MW (3)	gp
11	5	111.5	5.1 (-20)	pI/MW (4?)	T,S *
17		100	5.0		*
24	11	73.2	5.7 (-13b)		
26	13	71.5	4.5	pI/MW (4)	T,S
27		68.5	4.7	MW (2)	
35		63.1	5.1		*
48		52	5.1		R
61		44.2	6.4 (-3b)	pI/MW (3)	gp
64		46.6	4.6		gp
65		43.0	4.6		R,T
74		40.3	4.7		gp
80	27?	35.9	<4.5		*
82		35.1	4.7		*
89		33.4	6.4 (-3a)		S
104		27.3	5.9 (-11a)		gp?
110		26.3	6.3 (-6a)	pI (3)	
112		27.6	6.5 (-2a)	+/-?	T
127		24.1	6.3 (-5b)	+/-?	
132		20.1	5.6 (-15b)		*
134		20.7	6.3 (-6b)		S
135	34?	27.1	6.0 (-10b)	pI (2)	
x		85.1	4.8		*
a		104	5.0		ag
b		76.9	6.5 (-1b)		ag

(i) These proteins were characterised in extracts of SK17 except for those underlined which were analysed in experiments using T9 clones 32 or 94.

(ii) Equivalent proteins characterised by Tait (1981) and identified in fig. 3.1b.

(iii) pI's are estimated to nearest 0.1 pH unit. Where appropriate the identity of the nearest CPK charge isomer (see fig. 2.2) is given in brackets indicating whether the parasite protein is to the acidic (a) or basic (b) side of the isomer.

(iv) Proteins synthesised predominantly by rings, trophozoites or schizonts are denoted R,T or S respectively. Proteins marked * are preferentially lost during saponin lysis of infected erythrocytes. 'gp' and 'ag' indicate glycosylated and antigenic proteins respectively.

Appendix 3 Isolate typing by 2D gel analysis

Part of the results presented in Table 4.2, showing the variants of proteins A - M in different isolates, has previously been published in Walliker (1983, 1984). However, subsequent changes in the numbering of proteins and their variant forms have resulted in a number of apparent discrepancies between Table 4.2 and the earlier published work. For reference, these changes in nomenclature are listed below. Other differences (not given) between the two sets of data are the result of more detailed analyses of isolates or autoradiographs and, for purposes of strain typing, Table 4.2 supercedes the earlier versions.

Walliker (1983, 1984) Protein/variant	This thesis (Table 4.2) Protein/variant	#
B6	B9,10,11	1
E1	E1 or 2	13
E2	E4	"
H3 or 7	H3	50
I1	J1	86
I2	J2	"
-	I1	85
I3	I2	"
J	M	98
K3	K3,5 or 7	101